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IDENTIFICATION OF THE IMMUNE SYSTEM RESPONSIBLE FOR THE SPECIFICITY OF ACTIVELY ACQUIRED TOLERANCE IN MICE

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Communicated by Alexander Hollaender, December 17, 1962

Adult mice of one inbred strain injected at birth with hemopoietic cells from mice of another inbred strain can tolerate a skin graft from mice of the second strain. This unresponsiveness is antigenically specific, for such tolerant mice reject with normal vigor homologous skin from mouse strains unrelated to the donor of the neonatal inoculum.[1]

Mice tolerant to homografts can be considered cellular chimeras, since the detection of donor antigen several months after the neonatal injection of hemopoietic cells would suggest that donor cells can "take" and proliferate in the young mouse,[2] just as is known to occur spontaneously[3] or experimentally[1] in other species. Definitive evidence for the existence of cellular chimerism was presented by Trentin and Session[4] who were able to identify donor cells in tolerant mice by use of a chromosomal marker. Since it is well established that the tissues commonly used to induce tolerance, i.e., splenic tissue, contain antibody-forming cells, the occurrence of runt disease (interpreted as being due to a graft-antihost reaction) in tolerant mice of some donor-recipient strain combinations should indicate that immunologically competent donor cells can colonize the injected host.[5] Thus the possibility that tolerant mice have an immune system partially or completely of donor type raises the question of whether the immune response against unrelated antigens, which defines the specificity of tolerance, is due to the host or the transplanted cells. The solution of this problem should make it possible to determine whether the specificity of tolerance is a property of the host or donor immune system and, therefore, to establish whether the definition of tolerance so far referred to the whole animal[1] could also be applied to the host immune system.

Whether antibody-forming cells were of host or donor origin was previously investigated in mouse radiation chimeras of homologous constitutions. The immune system was shown to be exclusively of donor type.[6] A similar experimental design was used in the present work to identify the type (host or donor) of antibody-forming cells responsible for the immune response elicited in tolerant mice by antigens unrelated to the host and donor strains.

Materials and Methods.—Induction of tolerance: C3H/Anf Cum newborn mice of both sexes

were injected through the orbital branch of the anterior facial vein with 1×10^7 nucleated spleen cells in 0.05 ml volume from adult (3–4 months old) CBA/Cum females. The spleen cell suspension was prepared as previously described.[6] Two months after injection the survivors and normal C3H/Anf Cum mice of the same age were grafted with skin from adult CBA/Cum mice of the same sex as the recipient. The skin was obtained by splitting a donor ear into two layers, one layer of which was transplanted to each recipient. The grafting procedure used has been described by Billingham *et al.*[7] The survival time of the homologous skin was estimated from the outward appearance of the graft. Neonatally injected mice were considered tolerant when they retained a healthy homologous skin graft for a time significantly longer than did normal mice.

Sensitization of tolerant and normal mice to rat RBC: Five-month-old tolerant and normal mice were injected intraperitoneally with 1 ml of 1 per cent rat RBC. Twelve days later they were decapitated, and blood was collected individually for serum titration of antirat RBC agglutinins. A twofold serial dilution procedure was used. The titer was defined as the reciprocal of the highest dilution giving a macroscopic agglutination and converted to $\log_2$ (ref. 8).

Identification of the immune system of tolerant mice: The method used has been described in a previous paper.[6] Normal C3H/Anf Cum and CBA/Cum adult mice were preimmunized by intraperitoneal injection of 1×10^7 nucleated spleen cells from adult CBA/Cum or C3H/Anf Cum mice, respectively, and 10 days later were given a total-body exposure of 700 r. Within 2 hr after irradiation the preimmunized mice of each type were divided into four groups. A control group received intravenously 1 ml of Tyrode's solution. The others received intravenously one of the following inocula in 1 ml volume Tyrode's solution: 24×10^6 nucleated spleen cells from C3H/Anf Cum, CBA/Cum or tolerant C3H/Anf Cum mice. All donor mice had been sensitized against rat RBC 12 days earlier, and each cell suspension was prepared from a pool of 5–6 spleens.[6] Six days later, the recipients were decapitated, and blood was collected individually for serum titration of antirat RBC agglutinins. As schematically indicated in Table 1, the antirat RBC agglutinins detected in each type of recipient should be due only to the secondary response of the transferred spleen cells, and should not be detected if the recipients have been specifically preimmunized to the homologous spleen cell donors. Therefore, if tolerant mice sensitized against rat RBC are cellular chimeras, it should be possible to identify which type (host or donor) of immune system accounts for the agglutinin production.

Detection of donor antigenicity in tolerant mice: If tolerant mice are cellular chimeras, the injection of their tissues (containing donor-type antigens) into mice of the host strain should elicit transplantation immunity against tissues of the donor strain.[9] In the method to be described, this transplantation immunity was measured by the rejection of a challenge dose of donor-type bone marrow in lethally irradiated mice of the host strain injected, before irradiation, with spleen cells from tolerant mice. The bone marrow rejection was estimated by measuring Fe^{59} uptake in erythrocytes, using a technique described by Hodgson.[10] The challenge dose of bone marrow was determined by injecting intravenously 0.5 ml aliquots of a cell suspension of CBA/Cum bone marrow into groups of adult C3H/Anf Cum mice given a total-body exposure dose of 800 r 2 hr before. The bone marrow cell suspension was prepared as already described,[6] and the cell numbers given to the recipient groups ranged from 6.2×10^4 to 2×10^6. The recipient mice, 7 days after the treatment, were injected intravenously with ~ 0.2 μc of Fe^{59}. Radioactivity was measured in a blood sample taken 24 hr after injection of the isotope. As indicated in Figure 1, the iron uptake, which in the bone marrow noninjected group was 0.1 per cent of the Fe^{59} dose, increased linearly with the bone marrow dose up to 13.4 per cent. The corresponding cell number, 5×10^5, was chosen as a challenge dose.

The test for detection of donor antigens in tolerant mice consisted of the following procedure. Five to six tolerant mice, 5 months old, were decapitated, and a cell suspension was prepared from the pooled spleens. Ten million nucleated spleen cells in 1 ml volume were injected intraperitoneally into normal C3H/Anf Cum adult mice. Ten days later these and noninjected control mice of the same strain were given 800 r and 5×10^5 CBA/Cum bone marrow cells intravenously. Fe^{59} injection and bleeding followed as just described. Under these conditions any significant diminution of Fe^{59} uptake in the group of recipients injected with cells from tolerant mice, as compared to the control group, should indicate that CBA cells or antigens were present in the tissues of tolerant mice. The sensitivity of the method was determined by injecting C3H/Anf Cum mice before irradiation with different doses (from 10 to 1×10^7) of CBA/Cum spleen cells. The

TABLE 1

IDENTIFICATION OF THE IMMUNE SYSTEM OF TOLERANT MICE: EXPECTED SECONDARY AGGLUTININ RESPONSE IN IRRADIATED RECIPIENTS BY TRANSFERRED SPLEEN CELLS PRESENSITIZED AGAINST RAT RBC (THEORETICAL RESULTS)

	Spleen Cell Donors Sensitized against Rat RBC					
				Tolerant C3H		
				Possible types of immune system		
Irradiated recipients	No cells	C3H	CBA	C3H	CBA	C3H and CBA
C3H anti-CBA	0	+	0	+	0	+
CBA anti-C3H	0	0	+	0	+	+

specificity of the test was determined in a preliminary experiment in which C3H/Anf Cum mice preimmunized with 1×10^7 CBA spleen cells were challenged, after irradiation, with 5×10^5 C3H/Anf Cum bone marrow cells. No diminution of the Fe^{59} uptake was observed in this group as compared to the control group, which had not been pretreated with CBA/Cum cells.

Results.—Survival of skin grafts: CBA/Cum skin grafted on 12 C3H/Anf Cum 8-week-old mice showed complete necrosis in an average time of 11.7 ± 0.4 (standard error) days. All the 22 C3H/Anf Cum mice neonatally injected and skin-grafted when 8 weeks old showed a healthy CBA/Cum skin 3 months later (time of killing) and they were, therefore, considered to be highly tolerant.[1] None of the tolerant mice showed any sign of runt disease at any time before and after skin grafting.

Agglutinin production: Table 2 illustrates the agglutinin responses in tolerant mice and age controls 12 days after sensitization with rat RBC. Since the mean $\log_2$ titers of the two groups were not significantly different (P = 0.20), the data indicate the tolerant mice reacted with normal vigor against rat RBC.

Identification of the immune system of tolerant mice: Table 3 shows that the anti-rat RBC agglutinin titer measured in the serum of preimmunized and irradiated recipients was due only to the secondary response of spleen cells transferred from the sensitized donors. C3H/Anf Cum or CBA/Cum spleen cells did not produce agglutinins when transferred into irradiated recipients specifically preimmunized to the spleen cell donor. Spleen cells from tolerant C3H/Anf Cum mice produced agglutinins when transferred into irradiated C3H/Anf Cum mice preimmunized to CBA/Cum tissues, but they did not yield any agglutinin titer when transferred into irradiated CBA/Cum mice preimmunized to C3H/Anf Cum tissues. This finding demonstrates that antirat agglutinin production in tolerant mice was due to the activity of an immune system exclusively of host type.

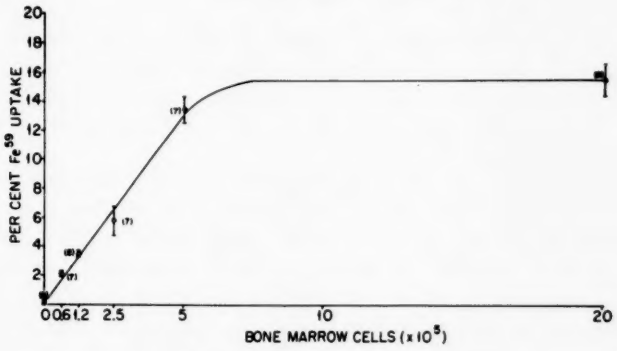

FIG. 1.

TABLE 2

AGGLUTININ RESPONSE AGAINST RAT RBC IN NORMAL AND TOLERANT MICE*

	Normal C3H	Tolerant C3H
$\bar{x}$	7.6	8.0
$s_{\bar{x}}$	0.2	0.3
No. of observations	10	11

* $\bar{x}$ = mean $\log_2$ titer; $s_{\bar{x}}$ = standard error.

TABLE 3

SECONDARY AGGLUTININ RESPONSE IN IRRADIATED (700 r) RECIPIENTS BY TRANSFERRED SPLEEN CELLS PRESENSITIZED AGAINST RAT RBC*

Irradiated recipients		No. cells	Spleen Cell Donors Sensitized against Rat RBC: C3H	CBA	Tolerant C3H
C3H anti-CBA	$\bar{x}$	0	10.5	0	8.9
	$s_{\bar{x}}$	0	0.2	0	0.1
	n	8	15	7	15
CBA anti-C3H	$\bar{x}$	0	0	10.4	0
	$s_{\bar{x}}$	0	0	0.2	0
	n	9	12	14	12

* See footnote, Table 2; n = number of recipients.

TABLE 4

Fe^{59} UPTAKE IN ERYTHROCYTES OF PREIMMUNIZED AND IRRADIATED (800 r) C3H RECIPIENTS GIVEN 0.5×10^6 CBA BONE MARROW CELLS*

	Preimmunizing Spleen Cell Dose: CBA 0	CBA 10^2	CBA 10^3	CBA 10^4	CBA 10^5	CBA 10^6	CBA 10^7	Tolerant C3H 10^7
$\bar{x}$	15.3	13.0	8.2	10.4	3.5	1.0	0.8	2.0	5.8
$s_{\bar{x}}$	0.6	2.3	1.6	0.7	2.0	0.2	0.2	0.6	1.8
n	23	8	6	8	6	7	4	5	9

* $\bar{x}$ = mean % Fe^{59} uptake; $s_{\bar{x}}$ = standard error; n = number of recipients.

Presence of donor antigenicity in tolerant mice: The results presented in Table 4 indicate that the spleens of tolerant mice contained cells or antigens of donor type. The transplantation immunity elicited by the donor component present in 1×10^7 spleen cells from tolerant mice was comparable to that produced by 1×10^4 CBA/Cum spleen cells.

It should be noted that the method used led to the detection of transplantation immunity induced by as few as 1×10^2 cells in a system where recipient and donor mice had identical alleles (K/K) at the *H-2* locus, which is the most important among histocompatibility loci.

Discussion.—The results presented indicate that C3H/Anf Cum mice made tolerant to CBA/Cum skin by neonatal injection of spleen cells from CBA/Cum mice were able to produce a normal amount of agglutinins against a challenge dose of rat RBC. This shows that the unresponsiveness induced in these mice to the homologous skin was a specific one.

The data demonstrate that the agglutinin response in these tolerant mice was produced by an immune system exclusively of host type, with the implication that immunologically competent donor cells either were absent at the time of the rat RBC challenge or, if present in sufficient number to be detected, were incapable of responding to this antigen. If immunologically competent donor cells were absent from the spleens of tolerant mice, they must have disappeared some time after the neonatal injection. Possible mechanisms of disappearance of donor cells might

be: (*a*) physiologic (nonimmunologic) competition in favor of host cells in colonizing the growing lymphatic tissues of the young mouse, a possibility suggested by Barnes *et al.*[11] to explain reversals to host type in cell populations of radiation chimeras or (*b*) self-destruction of donor cells during their reaction against the host, as shown by Boyse[12] to occur in some strain combinations. If the disappearance of donor cells occurred at a very early time, this condition might be sufficient to explain why no runt disease could be observed. Were immunologically competent donor cells present in the tolerant animal, they may not have been able to respond to rat RBC because of being tied up by an excess of host antigen. This possibility, implying a graft-antihost immune reaction, might also be in agreement with the absence of runt disease; the identity of host and donor at the *H-2* locus would suggest a very weak graft-antihost reaction, which does not produce gross evidence of runt disease.

Since in these tolerant mice the host immune system was the only one responsible for the normal agglutinin response against rat RBC, such an immune system could be considered to be in a tolerant state (specific unresponsiveness), because it was capable, while tolerating a homologous skin, of responding with normal vigor to antigens unrelated to the host and donor strains.

The fact that in the present work no immune activity of donor type could be detected might seem in disagreement with a study of Michie *et al.*[13] In some experiments, these investigators found that host- and donor-type cells from mice supposed to be *specifically* tolerant could induce spleen enlargement when transplanted into appropriate recipient mice. This result would suggest, on the basis of the common interpretation of Simonsen's G.V.H. assay,[14] that host- and donor-type cells potentially capable of an immune response were both present in some of the tolerant mice. However, since their mice had not been tested for the specificity of tolerance, it is difficult to predict which immune system would actually have responded in the tolerant animals, had they been challenged with antigens unrelated to the host and donor strains.

The detection of donor-type antigenicity indicates that cells or cell products of donor origin were present in the spleen of tolerant mice. In either case the original source could have been the neonatal inoculum or the skin graft. The possibility that cell products could have been responsible for the donor-type antigenicity is suggested by the observation that foreign proteins can last for a long time in animals made specifically unresponsive.[15] Whether the presence of donor antigens is a necessary condition for the maintenance of tolerance is as yet an unanswered question. While the work by Smith and Bridges[16] in rabbits, by Mitchison[17] in fowl, and by Trentin and Session[4] in mice, indicates that the tolerant status depends upon the continued presence of antigen, other experiments by Nossal[18] in rats and by Billingham and Silvers[19] in mice suggest that persistence of tolerance does not require persistence of the antigen. Further studies are needed to resolve this problem, fundamental to the understanding of tolerance.

Summary.—Tolerance to CBA/Cum skin was induced in C3H/Anf Cum mice by neonatal injection of CBA/Cum spleen cells. The tolerant status of these mice was a specific one, for they were able to produce a normal amount of agglutinins against a challenge dose of rat RBC. The immune system responsible for the specificity of tolerance in these mice was identified to be of host type. No immune ac-

tivity of donor cells against rat RBC could be detected, whereas donor type antigenicity was found in the spleen of the tolerant mice. On the basis of these findings it was proposed that the definition of tolerance given for the whole animal could also be applied to its immune system.

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† Operated by Union Carbide Corporation for the United States Atomic Energy Commission.

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*ILLEGITIMACY AND SPECIFIC FACTOR TRANSFER IN SCHIZOPHYLLUM COMMUNE**

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In *Schizophyllum commune*, a tetrapolar basidiomycete, the dikaryotization of a homokaryon by a dikaryon is expected if the dikaryon contains a nucleus that is compatible with the nucleus of the homokaryon, i.e., $(A^1B^1 + A^2B^2) \times A^3B^3 \rightarrow (A^1B^1 + A^3B^3)$ or $(A^2B^2 + A^3B^3)$, $(A^1B^1 + A^2B^2) \times A^2B^3 \rightarrow (A^1B^1 + A^2B^3)$.[1-3] The mating, $(A^1B^1 + A^2B^2) \times A^1B^2$, is considered noncompatible, however, since each of the two nuclei of the dikaryon contains one incompatibility factor in common with one in the nucleus of the homokaryon (either common *A* factors or common *B* factors). Occasionally, however, genetic recombination of mating type factors between the two nuclei of the dikaryon produces a nucleus that is compatible with the nucleus of the homokaryon, i.e., $(A^1B^1 + A^2B^2) \times A^1B^2 \rightarrow (A^2B^1 + A^1B^2)$,

an "illegitimate" dikaryotization. A number of such illegitimate dikaryotizations have been analyzed with respect to the mechanism of recombination.[4] The method for analysis was based on the recombination of several biochemical mutations in nuclei in which recombination for mating type factors had occurred. Two classes of recombinants were observed. Class I was characterized by possessing some combination of nonselective markers, biochemical mutations, present only in the original dikaryon. The recombinational event, therefore, involved only the two nuclei of the original dikaryon and was distinguished from the parasexual cycle described by Pontecorvo[5] by the high frequency of recombination between linked markers. Reduction was considered to be a meiotic event as the linkage relationships were very similar (with exceptions of two small subgroups) to those obtained via the standard sexual cycle. Class II individuals possessed, aside from mating type factors from the two nuclei of the original dikaryon, only genetic markers from the original homokaryon. A mating type factor specificity had apparently been transferred from each of the two nuclei of the original dikaryon into the nucleus of the homokaryon. Obviously, such an event involved all of the three original nuclear types.

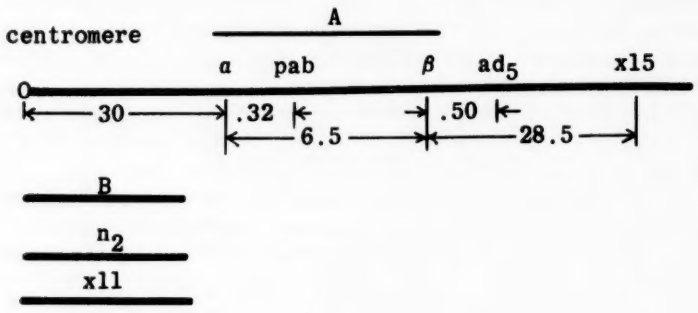
Three hypotheses were offered to explain the origin of Class II individuals.[4] (1) A triploid nucleus was formed from the three original nuclear types, underwent haploidization, and haploids with the same genetic markers as the original homokaryon were selected to dikaryotize the homokaryon. (2) Two successive recombinational events occurred, the first in the original dikaryon and the second in the derived dikaryon, and were followed by the selection of a nucleus homozygous, except for mating type, with the nucleus of the homokaryon. (3) There is a mechanism of genetic recombination that is specific in that only the incompatibility factors are recombined.

The first two hypotheses assume that there is strong internuclear selection based on different biochemical mutations and their wild-type alleles, an assumption that is not substantiated in studies of fully compatible dikaryotic-homokaryotic matings.[3] To obtain internuclear selection, there must have been a burst of recombinational events to provide a population of recombinants from which to select. No evidence is available that recombinational events produce populations of different types of recombinants within a single thallus. Support for the third hypothesis requires a demonstration that *only* the incompatibility factor specificities are transferred from the two nuclei of the dikaryon to the homokaryon.

The experiments reported herein were designed (1) to study the origin of Class II recombinants with the aid of two mutations, *pab* (para-aminobenzoic acid requiring), located between the two subunits of the *A* factor, and ad_5 (adenine requiring), located distal to *A* but very closely linked to $A\beta$, and (2) to prove conclusively the genotypes of the recombined nuclei incorporated into the derived dikaryons by several methods of analysis.

Materials and Methods.—All cultures used in this study were highly isogenic except for mating type factors (A^{41}, A^{42}, A^{47}, A^{d}, B^{41}, B^{42}, B^{47}) and biochemical mutations n_2 (nicotinic acid requiring), *x11* and *x15* (two unlinked mutations with unknown requirements), *pab*, and ad_5. Strain 699 was the background genome.[4]

A linkage map of the mutants used in this study is represented as follows:[6, 7]



All matings were made on migration-complete media.[8] Tests for specific nutritional requirements were made on minimal and appropriately supplemented media[9] or for the heterokaryotic allelic tests on minimal medium and migration-complete medium supplemented with para-aminobenzoic acid.

The illegitimate dikaryotic-homokaryotic matings were made as follows: The two homokaryotic cultures ($A^{41}B^{42}ad_5n_2$ and $A^{42}B^{41}pab$) that were used to synthesize the dikaryon were mated upon agar plates and incubated at 33°C; a dikaryon was synthesized within 72 hours. The homokaryon ($A^{41}B^{41}$ *x11 x15*) in the illegitimate matings was inoculated onto 5 plates and incubated at 22°C for 72 hr. The newly formed dikaryon ($A^{41}B^{42}ad_5n_2 + A^{42}B^{41}$ *pab*) was macerated with sterile distilled water, and a small aliquot of macerate was pipetted onto the growing edge of each of the five colonies of the homokaryotic culture, $A^{41}B^{41}$ *x11 x15*. The total process was repeated 240 times to make a total of 1,200 dikaryotic-homokaryotic matings. Forty-eight hours after the dikaryotic-homokaryotic matings were made, the dikaryon was removed by cutting out that portion from the plate. Seven days after the dikaryotic-homokaryotic matings were made, the plates were examined for the presence of dikaryotic sectors in the homokaryon. Hyphal tips of 1–3 cells were cut from dikaryotic sectors and placed upon migration-complete medium plus 0.2% yeast extract. The hyphal tips produced small colonies in five days at 22°C; the cultures were then stored at 1°C to prevent fruiting so that analysis of genetic constitution could be made later.

Experimental Results.—Eighty-six cultures were obtained from the isolation of hyphal tips from dikaryotic sectors that arose in the homokaryon in illegitimate dikaryotic-homokaryotic matings.

The first analysis of the 86 cultures consisted of mating each culture with four tester strains to determine the mating factor constitution of each of the two nuclei in each culture. The reaction patterns observed with the four testers, the frequency of occurrence of each pattern, and the genetic constitution necessary in the dikaryotic culture to elicit each pattern were as follows:

Genetic constitution of dikaryon	No. of cultures	Testers			
		$A^{41}B^{41}$	$A^{41}B^{42}$	$A^{42}B^{41}$	$A^{42}B^{42}$
$A^{41}B^{41} + A^{42}B^{42}$	22	+	−	−	+
$A^{41}B^{42} + A^{42}B^{41}$	42	−	+	+	−
$A^{x}B^{41} + A^{42}B^{42}$	4	+	+	−	+
$A^{41}B^{41} + A^{x}B^{42}$	2	+	−	+	+
$A^{42}B^{41} + A^{x}B^{42}$	8	+	+	+	−
$A^{x}B^{41} + A^{41}B^{42}$	3	−	+	+	+
$A^{x}B^{41} + A^{y}B^{42}$	3	+	+	+	+
$A^{41}B^{41} + A^{41}B^{42}$ (common A) ?	1	− (?)	−	+	+
$A^{42}B^{41} + A^{42}B^{42}$ (common A) ?	1	+	+	−	− (?)
Total	86				

+ = compatible reaction; − = noncompatible reaction.

Twenty-two cultures were $A^{41}\ B^{41} + A^{42}\ B^{42}$ and should represent dikaryons formed between the $A^{41}\ B^{41}$ nuclei of the homokaryon and the $A^{42}B^{42}$ nuclei resulting from recombination between the two types of nuclei of the original dikaryon, i.e., $A^{41}B^{42} + A^{42}B^{41} \rightarrow$ nuclear fusion (2n) $\rightarrow$ haploidization $\rightarrow$ haploid $A^{42}B^{42}$. Forty-two cultures were $A^{41}B^{42} + A^{42}B^{41}$ and represent the two nuclei of the original dikaryons that migrated through the homokaryon and displaced the homokaryon on its periphery. Twenty dikaryotic cultures possessed recombinant A factors in one or both nuclei; the scoring of two cultures was questionable.

The 22 dikaryons that were recombinant for mating type factors—and were not intra-A factor recombinants—were analyzed by heterokaryotic allelic tests for the presence of nonselective markers, the biochemical mutations present in the recombinant nucleus $A^{42}B^{42}$. Each of the dikaryons was mated with a series of five $A^{41}B^{41}$ testers, each carrying a different biochemical mutation. The $A^{42}B^{42}$ nuclei migrated into the $A^{41}B^{41}$ tester strains, and the new dikaryons were plated on minimal medium and on migration-complete medium supplemented with para-aminobenzoic acid. The procedure is exemplified in the analysis of dikaryon *63 × 11253:*

		Media	
		Minimal	Complete
$(A^{42}B^{42} + A^{41}B^{41}) + A^{41}B^{41} \rightarrow A^{42}B^{42} + A^{41}B^{41} \rightarrow$		+	+
$n_2?\ ad_5?\ pab?\ x15?$	$x11\ x15\ x11?$	$n_2 \rightarrow$	n_2
"	$\times A^{41}B_{x11}{}^{41} \rightarrow A^{42}B^{42} + A_{x11}{}^{41}B^{41} \rightarrow$	−	+
"	$\times A^{41}B_{x15}{}^{41} \rightarrow A^{42}B^{42} + A_{x15}{}^{41}B^{41} \rightarrow$	−	+
"	$\times A^{41}B_{pab}{}^{41} \rightarrow A^{42}B^{42} + A_{pab}{}^{41}B^{41} \rightarrow$	+	+
"	$\times A^{31}B_{ad5}{}^{41} \rightarrow A^{42}B^{42} + A_{ad5}{}^{41}B^{41} \rightarrow$	+	+

+ = growth; − = no growth.

The recombinant nucleus with mating type factors, $A^{42}B^{42}$, possessed, therefore, biochemical mutations, *x11* and *x15*, and the wild-type alleles of n_2, *pab*, and ad_5.

The genotypes for the recombinant nuclei and the frequency of their occurrence were:

Genotype of recombinant nuclei	No.
$A^{42}B^{42}\ n_2\ pab$	2
$A^{42}B^{42}\ x11\ x15$	20

Two of the 22 recombinants possessed biochemical mutations from the original dikaryon (Class I recombinants).[4] Twenty possessed, aside from mating type factors, only nonselected markers from the original homokaryon (Class II).[4] Though *pab* was located between the 2 subunits of $A^{42\ 6}_{3-5}$ in the nucleus of the original dikaryon, the A^{42}_{3-5} factor specificity was transferred, in the 20 Class II recombinants, to the basic genome of the original homokaryon without transferring either the mutant *pab* allele or the $x15^+$ allele distal to the A factor in relation to the centromere.

A basic assumption in the mating of the 22 dikaryons, $A^{42}B^{42} + A^{41}B^{41}$, with the five tester strains, $A^{41}B^{41}$, each with a different biochemical mutation, was that only the $A^{42}B^{42}$ nuclei migrated into the homokaryons. There is now good evidence that the $A^{41}B^{41}$ nucleus from the dikaryon will also migrate into the homokaryon although the two nuclei are noncompatible.[10] There was, therefore, a possibility

of mis-scoring the recombinant nucleus by the method, but reconstruction experiments indicate the probability of mis-scoring to be very small.

As a further test of the genetic constitution of the 22 dikaryons, each of the 22 was fruited and a sample of 16 spores/dikaryon was collected.

Sixteen haploid progeny of each of nine dikaryons (Class II) were plated on four different media: minimal supplemented with (1) niacin + adenine, (2) adenine + para-aminobenzoic acid, (3) niacin + para-aminobenzoic acid, and (4) niacin + adenine + para-aminobenzoic acid. None of the progeny of any of the nine dikaryons grew on either of these four media. The dikaryons thus appeared to be homozygous for either *x11*, *x15*, or both *x11* and *x15*.

The 16 haploids from each of the 22 dikaryons were analyzed for mating type factors by mating with four testers, $A^{41}B^{1}$, $A^{42}B^{1}$, $A^{1}B^{41}$, and $A^{1}B^{42}$. A^{41} segregated from A^{42}, and B^{41} segregated from B^{42} among the progeny of each of the 22 dikaryons, and segregation approximated a 1:1 ratio for both *A*'s and *B*'s. There was no evidence for trisomic ratios or segregation of "modifier" mutations.

A heterokaryotic allelic test was also applied to the haploid cultures in order to determine what mutations each possessed. Each of the 16 haploids from each of the 22 dikaryons was mated with five testers, $A^{47}B^{47}n_2$, $A^{47}B^{47}$ *x11*, $A^{47}B^{47}$ *x15*, $A^{d}B^{47}$ *pab*, and $A^{d}B^{47}ad_5$, and six days later the newly synthesized dikaryons were put on minimal and complete media. The procedure is diagrammed as follows:

Haploid mutations?	Testers		Media: Minimal	Media: Complete
"	× $A^{47}B^{47}$ n_2 →	dikaryon →	+	+
"	× $A^{47}B^{47}$ *x11* →	" →	−	+
"	× $A^{47}B^{47}$ *x15* →	" →	−	+
"	× $A^{d}B^{47}$*pab* →	" →	+	+
"	× $A^{d}B^{47}$ ad_5 →	" →	+	+

The haploid culture, therefore, contained mutations, *x11* and *x15*.

The haploids from each of the two dikaryons of Class I segregated alleles at the four loci: n_2, *x11*, *x15*, and *pab*. All haploid progeny from the 20 dikaryons of Class II possessed only *x11* and *x15*. All 20 dikaryons were, therefore, homozygous wild-type for n_2, *pab*, and ad_5 and mutant for *x11* and *x15*, in full agreement with previous tests.

The two dikaryons, $A^{41}B^{41} + A^{x}B^{42}$, were tested for growth on minimal medium because of the possibility they represented transfer of only one of the subunits of the *A* factor. Growth should have occurred on minimal medium if they were Class I recombinants and not if they were Class II recombinants. Both grew on minimal media.

Discussion.—The results presented here conclusively demonstrate that the Class II recombinants do possess the nonselective markers from the original homokaryon, and only those markers. From the results presented here and from a previous study,[4] with but one exception (which could possibly have been an error in scoring), there is no evidence that any genetic material other than the incompatibility factors is transferred to the background genome of the original homokaryon. This fact is particularly significant since *pab* is located between the two subunits of the *A* factor.

To explain the origin of Class II individuals on the basis of one or two typical recombinational events,[4] it is necessary to assume (1) a significant number of mul-

tiple crossovers, precisely located in the genome; (2) multiple crossing-over occurring in restricted portions of the total number of recombinational events; (3) a population of recombinational events; and (4) an eventual internuclear selection, based on biochemical mutations, that is absolutely specific for the biochemical mutations that were present in the original homokaryon. Such a system seems highly improbable, particularly since a single recombinational event that reassociates the *A* and *B* factors from the original dikaryon is all that is necessary to satisfy the minimal requirements for the dikaryotization of the homokaryon.

Mutation of the incompatibility factors as a possible explanation has been ruled out on the basis of three arguments:[4] (1) Mutations of the *A* and *B* factors exhibit a loss of function rather than a change in specificity,[11-13] (2) there would have to be simultaneous mutations at both subunits of the *A* factor, and (3) the mutations would have to be directed.

Quintanilha[14] had suggested that the exchange of mating type factors could be achieved if mitotic spindles coalesced as the nuclei divided and migrated through the homokaryon. Such a system would involve exchange of whole chromosomes and, as such, would give no explanation why other genetic markers on the same chromosome as the *A* factor were not transferred.

The origin of the Class II recombinants obviously involves all three nuclear types present in the dikaryotic-homokaryotic mating. If the transfer of genetic material is nonreciprocal, two steps would be postulated, namely, the release of genetic material from two nuclei and incorporation of specific genetic units into the third nucleus.

The normal genetic stability of the incompatibility factor specificities suggests that very special conditions must be necessary to effect specific factor transfer. Once the appropriate physiological state is achieved, both subunits of the *A* factor are always, or almost always, simultaneously incorporated into the third nucleus. Only one subunit specificity, however, need be transferred to effect a compatible reaction with the nucleus of the homokaryon. The incorporation of specific genetic material into the nucleus would have to assume a specificity of incorporation analogous to the incorporation of a temperate phage into a bacterium and the prophage's association with a specific site on a bacterial chromosome.[15-18]

An experimentally testable model for the transfer process is not immediately available.

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*THE DEPENDENCE OF VIABILITY EFFECTS CAUSED BY IRRADIATION ON THE TYPE OF MATING**

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Experiments to determine the spectrum of induced viability mutations in the X-chromosome of *Drosophila melanogaster* and their effects in heterozygotes have been carried out in this laboratory for the last year. The daughters of irradiated males were mated individually to either of two kinds of males. The progeny of these matings were scored and the proportions of the classes obtained for each sex indicated the viability of flies hemizygous and heterozygous for irradiated chromosomes. It was found that the penetrance of the induced mutations in the heterozygotes depended on the type of mating. It could be shown further that the differences were due to intraculture competition and to the partial "replacement"[1] of one class of flies by another.

Material and Methods.—Two stocks were used throughout these experiments: (*a*) A wild-type (+) stock, Qiryat Anavim, originating from flies collected in nature by Dr. E. Goldschmidt. This stock was moderately inbred but has been kept for some time in mass cultures. (*b*) A marked-balancer stock, Binscty, "*y* sc^{81} *B In49* ct^{n8} sc^{8}," which was kindly supplied to us by Dr. H. J. Muller of the University of Indiana. Both stocks were kept in mass cultures throughout the experiments with an occasional passage through a culture of 3–5 pairs of parents.

Flies were grown on medium prepared according to the formula of E. B. Lewis, seeded with Wagner's Y–2 yeast strain.[2] The cultures were kept in a constant temperature room at 25°C. On hot days, however, the temperature rose occasionally to 30°C.

Irradiations were performed at the Department of Physical Chemistry with a G. E. Maximar–200 X-ray machine. The machine was operated at 200 KVP 15 mA, with a 1 mm Al, 0.25 mm Cu filter at a dose rate of approximately 250 r/min for 8 min.

The experimental procedure was essentially identical in all repeats carried out, with only minor changes between them. Binscty females were mated to +-males and *vice versa*. The heterozygous females were backcrossed to both Binscty and

+-males (F_{x-1}). From this F_{x-1} mating Binscty/+ virgin females (F_x) as well as +-males (F_x) were collected. About half of the males were irradiated and mated immediately to Binscty/+ females for three days, after which the males were discarded. The other group of males was left unirradiated but otherwise was treated in a similar manner to the first one. The Binscty/+ daughters (F_{x+1}) were mated individually in vials to 2–3 males. These females were mated either to Binscty males (series I) or to +-males (series II). All F_{x+1} females of the irradiated experiments were mated to F_{x+1} males from the control experiments and *vice versa*. This procedure ensured that the irradiated autosomes were equally distributed among control and irradiation experiments. The progeny of these matings (F_{x+2}) were scored on three occasions: on the 10th, the 14th, and the 18th days after mating.

From some of the cultures of series I virgin F_{x+2} Binscty/+ females were mated to Binscty males from the same series (though not necessarily of the same culture). If possible, two such matings were performed per culture, each with three pairs of parents, but whereas one mating was carried out in a vial, the other was carried out in a bottle. The progeny of these matings (F_{x+3}) were scored as those of the previous generation.

Results.—Viability of flies hemizygous for irradiated chromosomes was determined by the proportion of +-males out of the total number of males. Viability of flies heterozygous for irradiated chromosomes was determined by the proportion of the corresponding females (Binscty/+ in series I and +/+ in series II) out of the total number of females. Six major experiments were carried out. The results are summarized in Table 1.

TABLE 1

CHROMOSOMES TESTED IN DIFFERENT REPEATS AND THE VIABILITY OF HETEROZYGOTES AND HEMIZYGOTES FOR IRRADIATED CHROMOSOMES AND CONTROLS

	Irradiation					Control				
	No. chromosomes	Total females	Total males	"Viability" Heterozygotes	"Viability" Hemizygotes	No. chromosomes	Total females	Total males	"Viability" Heterozygotes	"Viability" Hemizygotes
Series I										
a.	61	3671	3345	54.8	51.7	53	2193	1999	54.9	53.9
b.	48	2190	1699	56.2	47.1	25	1246	1219	58.0	60.5
d.	115	6170	5735	54.9	48.5	88	5382	5418	55.0	55.3
e.	14	1230	1145	54.7	51.4	6	457	461	48.4	49.5
f.	90	7188	6241	52.6	47.1	54	4900	4394	53.0	51.6
Total	328	20449	18165	54.2	48.7	226	14178	13491	54.3	54.2
Series II										
a.	77	5165	4128	51.4	56.5	91	4575	3662	49.2	58.6
b.	117	5422	4065	49.6	56.6	65	3097	2401	48.4	59.8
c.	185	11503	9749	51.5	54.7	122	7824	6934	50.5	58.0
d.	88	5003	3905	50.0	49.5	76	4860	4413	49.2	53.3
e.	110	9520	8074	51.0	51.1	79	6925	6033	51.0	53.1
f.	165	13043	10540	49.8	50.3	122	11492	10185	52.1	51.5
Total	742	49656	40461	50.6	52.7	555	38773	33628	50.6	54.7

Irradiation caused a decrease in the viability of hemizygotes, amounting to about 11% in series I and *ca.* 4% in series II, or an average decrease of some 8%. Only about one half the reduction was due to the effect of lethal mutations, the remaining decrease in viability was caused by nonlethal detrimental mutations (Table 2). A difference between the two series was also observed when the effects of the irradiation in the heterozygotes were compared. The irradiation decreased the viabil-

TABLE 2

AVERAGE VIABILITY OF FLIES HETEROZYGOUS AND HEMIZYGOUS FOR IRRADIATED CHROMOSOMES AND CONTROLS

	Heterozygotes		Hemizygotes	
	Irradiation	Control	Irradiation	Control
Series I				
Lethals included	54.2	54.3	48.7	54.2
Lethals excluded*	54.2	54.3	51.0	53.8
Number of lethal +-chromosomes			26	0
Per cent lethals			7.9 ± 0.47	–
Series II				
Lethals included	50.6	50.6	52.7	54.7
Lethals excluded*	50.6	50.6	53.5	54.7
Number of lethal +-chromosomes			30	0
Per cent lethals			5.2 ± 0.23	–

* Both lethals in the +-chromosome and in the Binscty-chromosome were excluded.

ity of females in series I by about 0.2–0.5%, while the effect was from "heterotic" in the early repeats to detrimental in the later repeats for the females of series II. We first suspected that the different contribution of lethals to the two series had been the source of these differences, since lethals are known to be usually only partially recessive.[1, 3, 4] Exclusion of the lethals from the comparison (Table 2), however, did not change the results for the heterozygotes (in series I) or even cause a slight reduction of the "heterotic" effect (in series II). This led us to assume that the differences between the two series were due to intraculture competition. The upper part of Table 3 gives the average number of flies per vial in the two

TABLE 3

AVERAGE NUMBER OF FLIES IN IRRADIATION AND CONTROL EXPERIMENTS

	Series I					Series II				
	Binscty/Binscty	Binscty/+	Binscty/Y	+/Y	Total	+/+	Binscty/+	Binscty/Y	+/Y	Total
Repeats a–d										
Irradiation	24.1	29.6	24.4	23.7	101.8	29.5	28.5	21.3	25.5	104.8
Control	23.7	29.4	23.0	29.0	105.2	28.5	29.0	21.0	28.1	106.7
Repeats e–f										
Irradiation	38.1	42.8	37.1	33.9	152.0	41.3	40.8	33.4	34.3	149.7
Control	42.3	47.0	39.3	41.6	170.2	47.4	44.3	38.6	42.0	172.3

series for both the irradiation and the control experiments in the first four repeats. The total number of flies in the control experiments was similar in both series, as was also the total number of flies per vial in the irradiation experiments of series II. In the irradiation series I, however, there were somewhat less flies per vial than in the controls. Further analysis showed that irradiation caused a reduction in the number of males hemizygous for irradiated chromosomes in both series. There was only a small change in the number of flies in classes not carrying irradiated chromosomes. There was, however, a difference between the two series in the frequency of females heterozygous for the irradiated chromosomes; whereas the number of Binscty/+ females did not decline in series I, the number of +/+ females in series II increased and "replaced" the decrease in the number of the +-males. This "replacement" effect might therefore have biased the results, giving the impression that the irradiation had a "heterotic" influence on the +/+ flies in series II. During the four repeats of the experiment the food preparation was improved, since it was found to contain too little water. With the improve-

ment there was the expected increase in the yield of flies per vial, accompanied, however, by a decrease in the "heterotic" effect. At this stage we improved further culture conditions and carried out two additional repeats of the experiment (*e* and *f*). The vials in these repeats could support a higher number of flies; accordingly, intraculture competition was more relaxed. There was not much excess of larvae to compete for the space left unexploited by the +-males carrying the irradiated chromosome and no "replacement" was detected (lower part of Table 3). In these repeats no "heterotic" effect was found in either series. Stern *et al.*[1] described such a replacement when comparing the viability of flies heterozygous for lethals with that of flies heterozygous for nonlethal chromosomes. Under crowded conditions the number of heterozygotes was higher in those cultures where the class of lethal males was completely missing. Goldschmidt and Falk[3] were able to demonstrate the partially dominant effect of "recessive" lethals, once the effect of overcrowding was eliminated.

That replacement was of importance in determining the "viability" in these experiments can be further seen from experiments in which flies from some of the cultures of series I were mated for another generation. In these matings three pairs of parents were put in a vial or in a bottle, thus producing more severe and more relaxed crowding conditions respectively. Only a limited number of cultures has thus far been tested. Table 4 gives the data of all cultures tested (upper part)

TABLE 4

VIABILITY OF FLIES GROWN UNDER DIFFERENT CROWDING CONDITIONS

	Irradiation				Control			
	No. chromosomes	No. flies	"Viability" Heterozygotes	"Viability" Hemizygotes	No. chromosomes	No. flies	"Viability" Heterozygotes	"Viability" Hemizygotes
All tested								
Vials	157	15945	57.7	52.9	102	10816	56.5	53.6
Bottles	87	22095	53.3	44.5	42	11355	54.3	51.5
Parallel tests only*								
Vials	63	6656	57.5	45.8	32	3234	55.1	51.4
Bottles	63	13973	53.7	44.1	32	8896	54.0	50.9

* Only chromosomes for which there was both a vial and a bottle were counted.

and those for which both a vial and a bottle were counted (lower part). It can be seen that in the irradiation experiments the viability was lower in bottles than in vials for both the hemizygotes and the heterozygotes. There was only a slight parallel decrease in the control experiments. The "heterotic" effect observed in the vials was obviously due to the fact that three pairs of parents per vial (though not per bottle) produced crowding sufficient to initiate replacement (Table 5). Thus, the more intraculture competition was relaxed, the more pronounced was the deleterious effect of the irradiation.

TABLE 5

AVERAGE NUMBER OF FLIES GROWN UNDER DIFFERENT CROWDING CONDITIONS

	Flies/culture				
	Binsc/Binsc	Binsc/+	Binsc/Y	+/Y	Total
Vials					
Irradiation	23.0	31.4	21.6	24.3	100.3
Control	23.7	30.9	23.9	27.6	106.0
Bottles					
Irradiation	64.6	73.6	64.2	51.6	254.0
Control	63.0	74.8	64.3	68.4	270.4

Discussion.—In a crowded culture of Drosophila only a fraction of the larvae present can emerge as imagoes. If there were various genotypes among the larvae, competition might determine the relative numbers of larvae of each genotype arriving at the imaginal stage. The relative number of flies of each genotype will therefore depend not only on their intrinsic viability but also on the interaction between genotypes. It is therefore obvious that similarity in the *total* number of flies per culture does not indicate similarity in competition. The control experiments of the two series described here might serve as an illustration: whereas the total number of flies in the two series was similar, there were less Binscty/Binscty flies in series I vials than +/+ flies in series II vials. This difference caused an increase in the number of all three remaining (identical) genotypes in series II over series I. When irradiation handicapped the viability of the Binscty/+ females and competition was severe, they could replace only partially the decline in the +/Y males. (Replacement seemed, however, to be more complete when three pairs of parents were used). In series II, replacement by the +/+ females was more complete, in spite of the reduction of viability caused by the irradiated X-chromosome they carried.

Differences in the frequency of lethals recovered, as depending on the type of females inseminated, were described by Hildreth and Carson.[5] They rejected the possibility that the differences were due to different crowding conditions since "the size of the corresponding populations in the Basc and Bv crosses were similar." If, however, the similarity in the number of offspring indicated only that there was overcrowding in both crosses, it is very possible that there were different degrees of crowding in the two crosses. The Basc crosses might have been more crowded with larvae than the Bv crosses and hence the heterozygotes for lethals had less chance to survive in them. The difference in the frequency of lethals observed by us in the two series must be due to another cause, since it was the father, who did not carry an irradiated chromosome, that differed in the two series. Nondisjunction in the F_{x+1} Binscty/+ females could obscure some lethals in series II, but not in series I; it was, however, too rare to affect the results considerably.[6]

In a study of the effect of irradiation on the viability of flies heterozygous for an irradiated chromosome, Wallace[7] found an increase in the ratio of the +/+ class to the CyL/Pm-reference class, when it had one irradiated chromosome. He found, however, that the total number of flies per culture was identical in the controls and the experimental series. In Table 7 of Wallace's paper some average numbers of flies of the different classes are given; it can be seen that in spite of the equality in the total number of flies, there was a decrease in the number of three of the genotypes in the irradiation experiments as compared to the corresponding control numbers. One might wonder, therefore, whether the increase in the fourth class of the X-rayed cultures—namely, that of +/+ flies—was due to replacement. This gets some support from the observation that the average number in the first count of these same cultures was higher in *all* classes of the irradiated cultures than in the controls. It seems that while both the irradiated cultures and controls produced more larvae than could reach the imaginal stage—giving ultimately similar numbers of flies—the X-rayed cultures had been less crowded and had therefore a higher rate of development. When competition acted, the +/+ flies could fill every space available better than other genotypes. The only factor which can be conceived as being common to all the classes in the irradiation experiments *versus* all classes of

the control experiments was that in the former one-eighth of the genetic background was irradiated, while no irradiated background was present in the controls; this might have caused the somewhat less crowded conditions in the cultures of the irradiated series. The +/+ genotype *per se* was neither better nor worse in the X-ray experiments, but intraculture competition gave it an advantage there. Later experiments of Wallace[8] were carried out with a technique very similar to that of series I described here. The males irradiated with 1000 r were mated, however, to Basc females. The main series were counted on the 14th day after mating. Crowding was less extreme in the irradiated series, as demonstrated by the higher rate of development there; more flies per vial were counted in all four classes of these cultures than in the controls. There was only some replacement. In a much smaller experiment, where crowding was increased (six pairs per bottle) and counts were done on the 17th day after mating, replacement clearly took place. This can be compared with one of our preliminary experiments in which also a Basc chromosome was used (instead of the Binsety used later) (Table 6). Replacement

TABLE 6

VIABILITY TESTS OF HETEROZYGOTES AND HEMIZYGOTES FOR IRRADIATED AND CONTROL CHROMOSOMES AS TESTED IN THE PROGENY OF BASC/+ FEMALES

Dose	Counted on day	Flies per Vial (calculated) Basc/Basc	Basc/+	Basc/Y	+/Y	Total	% Basc/+ females	% +/Y males
Wallace,[8] Table III								
1000 r	14th	22.5	30.8	22.3	27.7	103.3	57.8	55.4
Control	14th	22.0	29.8	21.8	27.5	101.0	57.5	55.8
Wallace,[8] Table IV								
2000 r	17th	54.0	70.6	56.3	65.3	246	58.7	53.6
Control	17th	52.0	69.9	55.2	72.3	248	58.0	56.7
Present work, preliminary series 1								
2000 r	18th	25.1	37.4	27.0	30.3	119.8	59.9	53.0
Control	18th	25.4	34.6	26.4	34.6	121.0	57.7	56.8
Present work, preliminary series 2		+/+	Basic/+	Basic/Y	+/Y			
2000 r	18th	32.3	32.0	22.2	32.3	118.8	50.2	57.1
Control	18th	35.0	32.0	24.4	34.0	125.4	52.1	57.6

took place in series 1, but not so much in series 2. Accordingly the radiation effect appeared to be heterotic in series 1 and deleterious in series 2. It must be concluded, therefore, that the "heterotic" effect—observed sometimes in cultures where progeny of irradiated flies are tested—is probably not due to an intrinsic higher viability of heterozygotes for induced mutations but is a result of intraculture competition relationships.

Summary.—The daughters of irradiated males and unirradiated control males were mated individually to two types of males. The viability of males hemizygous for the irradiated chromosomes and of females heterozygous for the irradiated chromosomes was determined together with the corresponding control values. While the viability of males hemizygous for irradiated chromosomes was always reduced, the viability of females heterozygous for irradiated chromosomes was either reduced or increased, depending on the type of mating. Intraculture competition differences were demonstrated to be responsible for the difference between matings. The "heterotic" effect was observed whenever the females heterozygous for the irradiated chromosome were able to replace the decline in the number of males

hemizygous for irradiated chromosomes. The observed "heterosis" was therefore spurious, in that it was not in fact the effect of a viability of the heterozygote superior to that of either homozygote. It is suggested that some previous reports on differences in mutability between types of crosses or on "heterosis" of flies heterozygous for induced mutations were also caused by intraculture competition.

We wish to acknowledge the capable assistance of Mrs. N. Himel throughout this work.

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*A DISCUSSION AND PROPOSALS CONCERNING FOSSIL DINOFLAGELLATES, HYSTRICHOSPHERES, AND ACRITARCHS, II**,†

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Family AREOLIGERACEAE n. fam.

Type genus: *Areoligera* Lejeune-Carpentier 1938.[1]

Diagnosis: Dorso-ventrally flattened dinoflagellate tests, circular to irregularly elliptical or triangular in dorsal or ventral view. Wall of central body smooth or with a few to many projecting surface structures in the form of apiculate granules or basically spinelike processes on the one hand, low ridges, folds, or membranous septa on the other, or both in combination. Processes are typically either nontabular or intratabular (single or in groups), but granulate tests may show some larger sutural granules. The tips of processes may be free or interconnected by either trabeculae or a membranous outer layer. Archeopyle exclusively apical with conspicuous sulcal notch that lies consistently away from the midline of ventral surface. Bilateral symmetry commonly suggested by larger processes.

Genera referable to the family: *Areoligera* Lejeune-Carpentier 1938, *Canningia* Cookson & Eisenack 1960, *Chiropteridium* Gocht 1960, *Circulodinium* Alberti 1961, *Cyclonephelium* Deflandre & Cookson 1955, *Membranophoridium* Gerlach 1961, *Tenua* Eisenack 1958.

Discussion: The family is distinguished from the Hystrichosphaeraceae by the nontabular or intratabular major processes and by the exclusively apical archeopyle. It is distinguished from both the Hystrichosphaeraceae and the Hystrichosphaeridiaceae by several features: (1) the dorso-ventral flattening, (2) the offset sulcal

notch, (3) a tendency for surface structures on dorsal and ventral surfaces to be developed to different degrees, and (4) a tendency toward a bilateral symmetry in the form and distribution of large processes.

The outline in dorsal or ventral view may or may not show a distinct but rounded apical prominence and one or two unequal antapical prominences that reflect apical and antapical horns. The dorsal and ventral surfaces are commonly unlike in convexity (ventral surface less convex) and in details of the size, form, and distribution of processes (see Fig. 4, Part I†). Processes tend to be reduced in number and length in the central portions of these surfaces, especially the ventral one. A tendency toward bilateral symmetry in the distribution of processes of similar size and form is usually recognizable (conspicuous in some heavily ornamented types). This quasi-symmetry of the processes contrasts with the asymmetrical position of the sulcal notch and the asymmetrically developed traces of two antapical horns.

The wall may appear one-layered or distinctly two-layered. The inner layer of a two-layered wall forms the capsule, which varies from smooth to spinose. The outer layer surrounding the capsule may be relatively featureless, may form outwardly radiating processes, or may be itself supported by processes that extend from the capsule. Processes may be hyaline or fibrous, solid or hollow, and may stand freely or have simple or intricate connections with adjacent processes (especially in a more or less meridional row along the sides of dorsal and ventral surfaces). The girdle and polar processes may be of somewhat different design than the rest. A typical dinoflagellate tabulation often can be inferred partially from the number and arrangement of processes, but in many forms its only suggestion is the typically zigzag archeopyle margin. The distinctive sulcal notch in the margin of the exclusively apical archeopyle lies consistently to the left side of the ventral surface.

Lejeune-Carpentier[1] discussed thoroughly the morphology of *Areoligera*, omitting only one significant point in her analysis. She did not mention the conspicuous opening, the apical archeopyle, that truncates the outline of most specimens (see Fig. 4A, Part I). Her drawings, however, show this truncation: for example, her Fig. 1 of *A. senonensis*, the type species, and Fig. 7 of *A. coronata*; her Fig. 5 appears to represent the operculum of the archeopyle in still another species, *A. medusettiformis*. (Compare Evitt, 1961, pl. 8, Figs. 13–15; pl. 9, Figs. 3–5.) Mr. Hans Gocht has examined the holotype of *A. coronata*, which Lejeune-Carpentier also studied, and reports (*in litt.*) that the zigzag rupture line is clearly developed. The same features are well shown in Gocht's[2] illustrations of the similar genus *Chiropteridium*.

Most described species that are referable to Areoligeraceae as conceived here fall into two groups:

1. In one group, typified by *Tenua hystrix*, Forma C of Evitt (1961, pl. 1, Figs. 18–22), *Cyclonephelium compactum* and *C. distinctum* (Deflandre and Cookson, 1955), *Canningia*, and *Circulodinium*, the surface is smooth or ornamented with many closely spaced, short, subequal elements, often with expanded or T-shaped tips and some interconnections. The wall may appear to consist of one layer. If two wall layers are apparent, the outer one is supported by processes that rise from the inner one. A tabulation is usually not clearly indicated.

2. In the second group, typified by *Areoligera* (see Fig. 4, Part I), *Chiropteridium*

and *Membranophoridium*, the often conspicuously intratabular processes or process-groups are relatively longer, larger, and fewer. It is in this second group that an approach toward bilateral symmetry of process arrangement may be striking. The ventral tabulation is usually not distinct because the processes in the central area are reduced. The determinable tabulation is 4′, 6″, 5‴ (6‴ probably), 1p, 1⁗. The wall is usually distinctly two-layered, one layer forming the processes and one the capsule.

In larger perspective, many of the differences in morphological details that distinguish typical Hystrichosphaeraceae and Hystrichosphaeridiaceae on the one hand from typical Areoligeraceae on the other seem to relate to just two fundamental morphologic differences. One difference concerns the degree of dorso-ventral flattening, accompanied by a lateral shift in the position of the sulcal notch. The nearly spherical (or ellipsoidal) Hystrichosphaeraceae and Hystrichosphaeridiaceae approach a radial (or axial) symmetry. Such slight bilateral arrangement of their surface structures as can be detected is related to a sagittal plane that passes through the inferred position of the sulcus. The Areoligeraceae, in contrast, have compressed bodies with the inferred sulcus (at least the sulcal notch that marks its apical end) off toward one side of the ventral surface. The bilateral arrangement of processes, which is often conspicuous, is not related to the position of this notch, but to a plane that passes through the centers of dorsal and ventral surfaces. These features already have been described in some detail[3] in connection with a comparison of *Areoligera* and *Systematophora*.

The second fundamental difference concerns the degree of variation in the distance between the inner wall of the cyst and the inner surface of the theca, as measured by the processes that are inferred to have supported the cyst against the theca. In the Hystrichosphaeraceae and Hystrichosphaeridiaceae the approximately equal length of all processes on a single specimen indicates a central body essentially concentric within the theca. In the Areoligeraceae the lengths of processes on a single specimen are graded in a manner suggesting that the central portions of the ventral and dorsal capsule surfaces were close to, or in contact with, the theca, whereas the lateral portions were more remote from it.

New Group: Acritarchs

Definition: Small microfossils of unknown and probably varied biological affinities consisting of a central cavity enclosed by a wall of single or multiple layers and of chiefly organic composition; symmetry, shape, structure, and ornamentation varied; central cavity closed or communicating with the exterior by varied means, for example: pores, a slitlike or irregular rupture, a circular opening (the pylome).

Discussion: Many taxa of fossils once assigned to the Order Hystrichosphaerida can now be referred to the Dinophyceae on the basis of definitive morphological criteria. Among these taxa are the genus *Hystrichosphaera* and the family Hystrichosphaeraceae, the taxa upon which the name Hystrichosphaerida was based. Left behind by this transfer is a "residue" of forms of unknown affinities for which the name Hystrichosphaerida is no longer appropriate. It is for this "residue" that I propose the name *acritarchs* (Latin, acritarcha; French, acritarche; German, Acritarch; Russian, акритарх). A name is desirable to facilitate communication

about these widespread, often abundant, and morphologically varied microfossils that cannot (at the present state of our knowledge) be more precisely identified. The name chosen implies no affinity with any other organisms and is not derived from the name of any taxon included in the group (*ακριτος*, uncertain, confused, and *ἀρχή*, origin).

Along with Deflandre[4, 5] and others, I believe that the acritarchs as defined here are a polyphyletic assemblage. At this moment of proposal the group comprises the former Hystrichosphaerida broadly construed, with the exception of those fossils identifiable as dinoflagellates. Some acritarchs are probably dinoflagellates that lack the minimum of morphological features required for positive recognition. Surely most of them are not dinoflagellates, but, in aggregate, represent a variety of life stages (e.g., eggs, cysts, mature tests) of assorted one-celled and higher organisms, both plants and animals. Accordingly, and in contrast to the Hystrichosphaerida which was proposed as an order, this group is proposed as an informal, utilitarian, "catch-all" category without status as a Class, Order, or other suprageneric unit (no matter whether the vernacular or the Latin form of the name is used). Whenever the biological affinities of individual acritarch genera can be established with sufficient precision, those genera should forthwith cease to be referred to as acritarchs and should be assigned to their proper places in the taxonomic hierarchy under the appropriate nomenclatural code. For example, almost immediate removal of at least *Tasmanites* to the Chlorophyceae seems warranted.[6] As remarked earlier in this paper, I recommend that the acritarchs be treated under the Botanical Code.

In the sense proposed here, the acritarchs include some of the microfossils from the Huronian Gunflint formation, all Paleozoic ex-hystrichospheres, and many ex-hystrichospheres from the Mesozoic and Tertiary. However, most fossils from the last two eras that have been termed hystrichospheres in the past appear to be dinoflagellates. The following list includes the principal genera I would refer to the acritarchs until their biological affinities can be recognized: *Acanthodiacrodium* Tim. 1958 em. G.&M. Defl. 1962, *Anthatractus* Denuff 1954, *Antrosphaera* Sarjeant 1961, *Archaeohystrichosphaeridium* Tim. 1959, *Aremoricanium* Deunff 1955, *Baltisphaeridium* Eis. 1958, *Ceratocystidiopsis* Defl. 1937, *Cirrifera* Cook. & Eis. 1960, *Crassosphaera* Cook. & Manum 1960, *Cymatiogalea* Deunff 1961, *Cymatiosphaera* O. Wetzel 1933 em. Defl. 1953, *Dasydiacrodium* Tim. 1959 em. G. & M. Defl., 1962, *Deunffia* Downie 1959, *Dictyosphaeridium* W. Wetzel 1952, *Dictyotidium* Eis. 1955, *Dioxya* Cook. & Eis. 1958, *Diplofusa* Cook. & Eis. 1960, *Diplotesta* Cook. & Eis. 1960, *Disphaeria* Cook. & Eis. 1960, *Disphaerogena* O. Wetzel 1933, *Domasia* Downie 1959, *Estiastra* Eis. 1959, *Fromea* Cook. & Eis. 1958, *Gillinia* Cook. & Eis. 1960, *Kalyptea* Cook. & Eis. 1960, *Komewuia* Cook. & Eis. 1960, *Korojonia* Cook. & Eis. 1958, *Leiosphaeridia* Eis. 1958, *Leiosphaeridium* Tim. 1959, *Lophodiacrodium* Tim. 1958 em. G. & M. Defl. 1962, *Lophosphaeridium* Tim. 1959, *Lunulida* Eis. 1958, *Micrhystridium* Defl. 1937, *Multiplicisphaeridium* Staplin 1961, *Netrelytron* Sarjeant 1961, *Omatia* Cook. & Eis. 1958, *Orycmatosphaeridium* Tim. 1959, *Palaeostomocystis* Defl. 1935, *Palaeotetradinium* Defl. 1934, *Platycystidia* Cook. & Eis. 1960, *Pleurozonaria* O. Wetzel 1933, *Polyedryxium* Deunff 1954, *Priscogalea* Deunff 1961, *Priscotheca* Deunff 1961, *Protoleiosphaeridium* Tim. 1959, *Pterocystidiopsis* Defl. 1935, *Pterospermopsis* W. Wetzel 1952, *Pulvinosphaeridium* Eis. 1954, *Sam-*

landia Eis. 1954, *Symplassosphaeridium* Tim. 1959, *Tasmanites* Newton 1875, *Trachydiacrodium* Tim. 1959 em. G. & M. Defl. 1962, *Trematosphaeridium* Tim. 1959, *Trigonopyxidia* Cook. & Eis. 1961, *Tytthodiscus* Norem 1955, *Vavososphaeridium* Tim. 1959, *Veryhachium* Deunff 1954, *Vulcanisphaera* Deunff 1961, *Zonosphaeridium* Tim. 1959. The shapes of some of the post-Paleozoic fossils included in the above genera suggest they may well be dinoflagellates, but other morphological characters that would substantiate their dinoflagellate affinities have not been noted.

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† For Part I, *see* these PROCEEDINGS, vol. 49, p. 158.

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*RESTRICTED INFRAPOLYNOMIALS AND TRIGONOMETRIC INFRAPOLYNOMIALS**

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The object of this note is (*i*) to present a complement to a theorem recently proved by the present writer and (*ii*) to indicate that the enlarged theorem applies to the study of trigonometric infrapolynomials. The result previously established[1] is

THEOREM 1. *Let the compact point set E containing at least* n *points lie on the circle* $|z| = 1$, *and let* A_n *be prescribed, with* $|A_n| = 1$. *Then all zeros of every restricted infrapolynomial* $p_n(z) \equiv z^n + a_1 z^{n-1} + \cdots + a_{n-1}z + A_n$ *on E with prescribed constant term* A_n *lie on* $|z| = 1$.

For the terminology the reader may refer to reference 1. We shall indicate the proof of the

COROLLARY. *Under the conditions of Theorem* 1, *every arc of* $|z| = 1$ *containing two zeros of* $p_n(z)$ *contains at least one point of E.*

That is to say, the zeros of $p_n(z)$ are weakly separated on $|z| = 1$ by a subset of E.

Let an arc α of $|z| = 1$ contain two zeros z_1 and z_2 (not necessarily distinct) of $p_n(z)$, but contain no point of E; we shall reach a contradiction. The polynomial

$p_2(z) \equiv (z - z_1)(z - z_2)$ is a factor of $p_n(z)$, hence (*loc. cit.*, Theorem 1) also an infrapolynomial on E with prescribed constant term. By a rotation of the plane if necessary we may suppose $p_2(z) \equiv z^2 - Az + 1$. Then (*loc. cit.*, Theorem 2) there must be satisfied the condition $[\min x] \leqq A/2 \leqq [\max x]$, where $z = x + iy$ ranges over all points of E, and of course we have $A = 2x_1$ with $z_1 = x_1 + iy_1$, $z_2 = x_1 - iy_1$. There follows $[\min x] \leqq x_1 \leqq [\max x]$, so α must contain at least one point of E, contrary to our assumption. The Corollary is established.

It may be pointed out that the infrapolynomial $p_n(z)$ of Theorem 1 on E: $|z| = 1$ or on a subarc of $|z| = 1$ need not have distinct zeros on $|z| = 1$. For instance $p_2(z) \equiv (z - 1)^2$ is such a polynomial on $|z| = 1$, because $p_2(z)$ cannot have an underpolynomial $q_2(z) \equiv z^2 - A_1z + 1$; in fact the inequality $|q_2(z)| = |z^2 - A_1z + 1| < |(z - 1)^2|$ cannot hold throughout a deleted neighborhood of $z = 1$, $q_2(z) \not\equiv p_2(z)$.

We turn now to the brief consideration of trigonometric polynomials. By analogy with algebraic polynomials, we say that $t_n(\theta) \equiv \Sigma_{k=0}^{n}[A_k \cos k\theta + B_k \sin k\theta]$ has a *trigonometric underpolynomial* $s_n(\theta) \equiv \Sigma_{k=0}^{n}[A_k' \cos k\theta + B_k' \sin k\theta]$ on a set E provided $A_n' = A_n$, $B_n' = B_n$, $A_n^2 + B_n^2 \neq 0$, $s_n(\theta) \not\equiv t_n(\theta)$, and provided we have

$$|s_n(\theta)| < |t_n(\theta)| \text{ on } E \text{ where } t_n(\theta) \neq 0, \tag{1}$$

$$s_n(\theta) = t_n(\theta) \text{ on } E \text{ where } t_n(\theta) = 0. \tag{2}$$

Here we assume A_n and B_n real, but not A_k, B_k, A_k', B_k' for $k < n$; we also assume the points of E to be defined by real values of θ. If $t_n(\theta)$ has no underpolynomial on E, it is called a *trigonometric infrapolynomial* on E.

By use of Theorem 1 and the Corollary we shall prove

THEOREM 2. *Let* $\mathrm{t}_n(\theta)$ *be a trigonometric infrapolynomial on a set* E *consisting of at least* 2n *points distinct* (mod 2π). *Then all zeros of* $\mathrm{t}_n(\theta)$ *are real, and* (*with* $\mathrm{w} = \mathrm{re}^{i\theta}$) *every arc of* $|\mathrm{w}| = 1$ *containing two zeros of* $\mathrm{t}_n(\theta)$ *contains at least one point of* E.

By the Euler formulas we may write with $w = re^{i\theta}$ and $r = 1$

$$\begin{aligned} 2t_n(\theta) &\equiv A_n(w^n + w^{-n}) - iB_n(w^n - w^{-n}) + \cdots \\ &\equiv (A_n - iB_n)w^n + \cdots + (A_n + iB_n)w^{-n}, \end{aligned}$$

where the terms not written involve w or w^{-1} to a nonnegative power less than n; since A_n and B_n are real, we have $|(A_n - iB_n)/(A_n + iB_n)| = 1$. On $|w| = 1$ there follows $2|t_n(\theta)| \equiv |(A_n - iB_n)w^{2n} + \cdots + (A_n + iB_n)|$. The condition that this last polynomial divided by $(A_n - iB_n)$ be an algebraic infrapolynomial with prescribed constant term on E is precisely the condition that $t_n(\theta)$ be a trigonometric infrapolynomial on E, where we allow comparison of $t_n(\theta)$ with underpolynomials $s_n(\theta)$ having possibly non-real coefficients (except for A_n and B_n). Theorem 2 follows from Theorem 1 and the Corollary.

With the hypothesis of Theorem 1 we choose B so that $B^2 = \overline{A}_n$, whence $B\bar{B} = 1$, and write

$$P_n(z) \equiv Bp_n(z) \equiv Bz^n + Ba_1z^{n-1} + \cdots + Ba_{n-1}z + \bar{B}.$$

Since all zeros of $p_n(z)$ lie on $|z| = 1$, for every such zero we have

$$\begin{aligned} 0 &= B\bar{z}^{-n} + Ba_1\bar{z}^{-n+1} + \cdots + Ba_{n-1}\bar{z}^{-1} + \bar{B}, \\ 0 &= \bar{B} + \bar{B}\bar{a}_1z + \bar{B}\bar{a}_2z^2 + \cdots + \bar{B}\bar{a}_{n-1}z^{n-1} + Bz^n. \end{aligned}$$

so this last equation is $0 = P_n(z)$. Consequently for the coefficients of $P_n(z)$ and $p_n(z)$ we have $Ba_1 = \bar{B}a_{n-1}$, $Ba_2 = \bar{B}a_{n-2}$, $\cdots$, $Ba_{n-1} = \bar{B}a_1$, equations involving some redundancy. If we apply this remark to the polynomial

$$(A_n - iB_n)w^{2n} + b_1w^{2n-1} + \cdots + b_{2n-1}w + (A_n + iB_n)$$

previously considered, with suitable adjustment of coefficients, further use of the Euler formulas shows that *for the trigonometric infrapolynomial $t_n(\theta)$ all coefficients are real.*

It is a consequence of Theorem 2 that trigonometric polynomials of the form prescribed for $t_n(\theta)$ which are extremal in the sense of Tchebycheff with weight function or in the sense of weighted least pth powers ($p > 0$) interpreted by summation for finite E or Stieltjes integration for infinite E—all such extremal trigonometric polynomials share the properties of the conclusion of Theorem 2.

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[1] *Jour. de math. pures et appl.*, *37* (1958), 295–316.

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*EVIDENCE OF CONTINUOUS TRANSMISSION OF NONINFECTIOUS SV 40 VIRAL GENOME IN MOST OR ALL SV 40 HAMSTER TUMOR CELLS**

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Extensive, critical investigations have provided no support for the hypothesis that malignant tumor cells induced in mice and hamsters by the DNA polyoma virus may retain the viral genome in a noninfectious state either in the form of "provirus," incomplete virus, or as vegetative viral nucleic acid incapable of maturation in the malignant cells.[1, 2] It has been established, however, that in the process of malignant transformation a new heritable character appears, in the form of an antigen (or antigens) that is not related to the antigens in polyoma virus but is, nevertheless, specific for this virus in the sense that isologous spontaneous or chemically induced tumors do not contain it[3–6] and that other viruses, including the tumor-producing DNA, SV 40 and adenovirus type 12, will not give rise to it.[7] During the course of our work on the demonstration of a similarly specific cellular antigen in SV 40 virus-induced transplantable hamster tumor cells,[7] it at first appeared that the SV 40 tumor cells were also incapable of yielding infectious virus. However, the occasional fleeting appearance of trace amounts of SV 40 virus in tissue cultures of the transplantable SV 40 tumors led to a series of investigations, to be reported here, which provided evidence for the continuous transmission of noninfectious SV 40 viral genome in most or all of the SV 40 tumor cells.

Materials and Methods.—Tumor and procedure for transplantation: An SV 40 virus-induced hamster fibrosarcoma[8] in the 16th transplant passage was obtained from Dr. Bernice Eddy. Serial

transplantation in this laboratory was performed by subcutaneous inoculation of 0.1 gm of finely minced, nonnecrotic tumor tissue—0.5 ml of a 20 per cent suspension in Eagle's minimum essential medium (MEM) + 10 per cent calf serum—in untreated random inbred Syrian female hamsters, weighing about 70 gm, obtained from a local breeder. The tumors became palpable in about a week, and were again passaged when they reached a size of about 40 × 30 × 15 mm at 2–3 weeks after inoculation. When small numbers of cells were used for transplantation, the tumors or tissue cultures prepared from them were trypsinized and the washed sedimented cells were resuspended in MEM medium + 10 per cent calf serum. After the larger clumps had settled out, the upper $^2/_3$ of the suspension was used, the cells being counted in a hemocytometer. Approximately 95 per cent of the cells were viable by the trypan blue test. The suspension was t hen further diluted in MEM medium + 10 per cent calf serum to obtain the desired number of cells in 0.1 ml for subcutaneous inoculation.

Propagation of tumor cells in tissue culture: Approximately $10^{7.3}$ trypsinized cells in 40 ml of MEM (0.22 per cent $NaHCO_3$) + 30 per cent calf serum were planted in 32 ounce bottles, which were incubated at 37°C. The medium was changed every 2–3 days regardless of the pH. Cellular degeneration occurred when the concentration of calf serum was less than 30 per cent, or the medium was not changed at the indicated intervals. When a heavy cell sheet had formed, usually 12–18 days after planting, the trypsinized cells from one bottle were planted in two bottles of similar size.

Tests for SV 40 virus: The stable line of Cercopithecus monkey kidney cells, BS-C-1,[9] was used. The stock cultures were maintained by planting about 10^7 trypsinized cells in 40 ml of medium 199 (0.125 per cent $NaHCO_3$) containing 0.1 per cent yeastolate and 20 per cent fetal calf serum in 32 ounce bottles. The medium was changed two days and five days after planting and passages were made on the seventh day. For preparation of roller tubes approximately $10^{5.3}$ trypsinized cells in 2 ml of the medium mentioned above were planted per tube. The medium is changed at two days, and on the fifth or sixth day, when an extensive sheet is usually present, the old medium is replaced by 2 ml of maintenance medium—199 (0.25 per cent $NaHCO_3$) + 2 per cent regular calf serum. The cells remain in good condition for 40 days or longer when the medium is changed weekly. In tests for virus the culture tubes are kept in a roller drum, and observations for cytopathogenic effect (CPE) are continued for 40 days because at the endpoint CPE sometimes appears as late as 37 days. The SV 40 virus produces a typical vacuolating CPE which progresses until all the cells are destroyed. A comparative titration indicated that the titer in BS-C-1 cells observed for 40 days can be about ten times higher than in primary Cercopithecus monkey kidney cells observed for the same number of days, but the latter cells are in poor condition at the end of three weeks. Titration of SV 40 virus in BS-C-1 tubes kept in a roller drum is also somewhat more sensitive than by the plaque technique in the same cells. Using ten tubes per dilution, the titer of the same preparation was $10^{7.7}$ TCD_{50} per ml. in the roller tubes and $10^{7.3}$ PFU per ml by the plaque technique, the plaques being counted 16 days after inoculation. In testing for virus in tumors a 15 per cent suspension in medium 199 (0.125 per cent $NaHCO_3$) was prepared in an "Omnimix" homogenizer, the homogenate was frozen and thawed once, centrifuged at 2,000 rpm for 20 min, and the supernatant fluid was stored at −70°C until it was tested. Usually 0.2 ml was added per tissue culture tube containing 2 ml of maintenance medium. Incubation was at 37°C in roller drums. The medium was replaced 24 hr after inoculation, in order to remove any hypothetical inhibitor, and then weekly thereafter. The frozen and thawed cultures and fluid media were tested similarly. The tubes were inspected microscopically just before each weekly medium change, so that those exhibiting a questionable or slight CPE could be changed last and separately with individual pipettes. Separate pipettes were used for changing medium in tubes without CPE belonging to any one specimen or dilution. Care was taken to avoid possible contamination from one set of tubes to another during the medium changes. Virus recovered from tumors or tumor cell cultures was identified as SV 40 by neutralization with anti-SV 40 virus rabbit serum.

Fluorescent antibody tests for SV 40 viral antigen: Tumor cells grown on cover slips in MEM + 30 per cent calf serum were washed five times (at least twice with 1 hr leaching periods) with serum-free medium 199 (0.125 per cent $NaHCO_3$), dried in air at room temperature, and fixed in acetone for ten minutes. The SV 40 immune rabbit serum, kindly supplied by Dr. M. Hilleman, was prepared with virus grown in washed, primary Cercopithecus monkey kidney cells maintained in serum-free

medium 199, and had a neutralizing antibody titer of 1:4,000 versus 100 TCD_{50} of virus. For control we used normal rabbit serum as well as polyoma immune rabbit serum prepared with virus grown in mouse embryo tissue cultures maintained in BME + five per cent horse serum. The indirect method was used, adding potent fluorescein-conjugated absorbed chicken anti-rabbit globulin to the specimens treated with the indicated rabbit sera. The effectiveness of the reagents and of the procedure was checked by finding very bright specific nuclear fluorescence in BS-C-1 cells infected with SV 40 virus but not in uninfected controls.

Results.—Irregular appearance of minute amounts of infectious SV 40 virus in occasional tumors during serial transplantation: Girardi *et al.*[10] reported that extracts of primary tumors developing many months after injection of large amounts of SV 40 virus in newborn hamsters yielded little or no infectious SV 40 virus, and that tests on three first passage transplant tumors yielded only minute amounts of virus. The report of these investigators[10] that frozen sections of 11 tumors nevertheless exhibited SV 40 viral antigen by the indirect fluorescence antibody technique is open to question because the fluorescence was not in the nuclei, where the viral antigen is produced. Our own preliminary tests on $10^{5.6}$ frozen and thawed trypsinized cells from a 16th transplant passage tumor and on a 15 per cent extract from a 19th transplant passage tumor yielded no virus. The data shown in Table 1 indicate, however, that during the course of serial transplantation of large numbers

TABLE 1

IRREGULAR APPEARANCE OF MINUTE AMOUNTS OF INFECTIOUS SV 40 VIRUS IN OCCASIONAL SV 40 HAMSTER TUMORS DURING SERIAL TRANSPLANTATION

Transplant passage no.	Hamster 1		Hamster 2		Hamster 3		Hamster 4	
	Interval[a] (days)	Result	Interval (days)	Result	Interval (days)	Result	Interval (days)	Result
19	23	0/5[b]	..	...	..	...	..	...
21	53	**10/10**[c]	..	...	..	...	..	...
22	36	**5/10**[d]	..	...	..	...	..	...
23	33	**5/9**	..	...	..	...	..	...
24	19	0/10; **3/10**; 0/10[e]	..	...	..	...	..	...
25	29	0/10; 0/10; 0/10[e]	70	0/10; 0/10[e]	77	0/20	..	...
26	16	0/10; 0/10; 0/10[e]	57	0/10; 0/10[e]	..	...	..	...
27	48	**7/16**	..	...	..	...	..	...
28	22	0/10; 0/10[e]	29	**1/20**	41	**4/10**	58	**7/10; 0/18**[f]
29	19	0/10	40	0/10	40	**19/20**	..	...
30	19	0/20	23	0/20	37	**8/20**	37	**19/20**
31	22	0/10	28	0/19	28	**8/20**	34	0/20

[a] Time between transplantation and removal of tumor; palpable tumor usually present within 7 days after transplantation.
[b] Numerator = number of tissue culture tubes exhibiting typical SV 40 cytopathogenic effect; denominator = number of tubes inoculated.
[c] Quantitative test yielded 1,000 PFU per gm of tumor.
[d] 33 TCD_{50} per gm of tumor.
[e] Test on sediment obtained by centrifugation at 30,000 rpm (79,420 × g) for 3 hr.
[f] Test on metastatic tumor in pleural cavity; tumor measured 20 × 15 × 15 mm.

of cells occasional tumors irregularly yielded minute amounts of virus between the 21st and 31st transplant passages. In some of these passages (24th–28th) an attempt was made to get rid of a hypothetical inhibitor by centrifuging extracts from 0.75 to 1 gm of tumor tissue at 30,000 rpm for 3 hr and testing only the sediment, which should contain the virus, but invariably with negative results. Although five tumors from the 25th and 26th passages failed to yield virus on repeated tests, minute amounts of virus were again recovered from 8 of 17 tumors in the 27th to the 31st passages. It should be noted here that no SV 40 virus neutralizing antibody, by either the standard or the plaque reduction techniques, was found in 12 tumor-bear-

ing hamsters, regardless of whether their tumors did or did not yield infectious virus.

Absence of SV 40 virus, except on rare occasions, in fluids from primary tissue cultures of SV 40 tumor cells: In the experiments summarized in Table 2, $10^{5.7}$ trypsinized tumor cells were planted on cover slips (50 × 10 mm) in each of a series of Leighton tubes, to which were added 2 ml of MEM medium + 30 per cent calf serum. Incubation was at 37°C, and the medium was completely replaced every two days. The fluids removed from all tubes on a given day were pooled, frozen at

TABLE 2

ABSENCE OF SV 40 VIRUS, EXCEPT FOR TRACES ON RARE OCCASIONS, IN FLUIDS FROM PRIMARY TISSUE CULTURES CONTAINING 2–5 × 10^5 SV 40 TUMOR CELLS IN LEIGHTON TUBES

Transplant passage no. of tumor	Virus in original tumor	Test for virus in culture fluids removed on indicated day						Fluorescent antibody test for virus in cells on indicated days
		2	4	6	8	10	12	
17—10^3 cells	Not tested	0/12[a]	**2/12**	0/12	0/12	..	..	Negative 14
17—10^2 cells	**10/10**	0/5	0/5	0/5	0/5	0/5	0/5	Not tested
17—10^1 cells	0/10; **2/10**[b]	0/5	0/5	0/5	0/5	0/5	0/5	Negative 4, 6, 8, 10
21—20% susp.	**10/10**	0/5	**3/5**	0/5	0/5	0/5	0/5	Negative 2, 4, 8, 12
22—20% susp.	**5/10**	0/5	0/5	0/5	0/5	0/5	0/5	Negative 2, 4, 8, 12

[a] Numerator = number of tissue culture tubes exhibiting typical SV 40 cytopathogenic effect; denominator = number of tubes inoculated.
[b] Metastatic tumor in axillary lymph node measuring 25 × 22 × 12 mm; was not used for the tissue cultures.

−70°C in an electric refrigerator, and aliquots were tested for virus. All the cells did not attach to the glass, so that the 2-day fluids contained many tumor cells. Within 2–4 days there was a confluent sheet of cells varying in number from $10^{5.3}$ to $10^{5.7}$. At intervals indicated in Table 2, some of the cover slips were fixed for detection of SV 40 viral antigen by the fluorescent antibody technique. In the five experiments listed in Table 2, among a total of 28 fluid changes tested only two—both of them oddly enough only on the fourth day—yielded traces of virus. In two experiments in which the original tumor contained demonstrable infectious virus, none of the fluids harvested over a period of 12 days contained any demonstrable virus. Moreover, no SV 40 viral antigen was detected by the fluorescent antibody technique in the nuclei of the cells on the cover slips. Thus, while there was no evidence of progressive viral multiplication or of the formation of viral antigen in the tumor cells in tissue culture, there was an indication in two experiments that an occasional cell (or cells) may spontaneously yield a small amount of infectious virus without setting off a consecutive chain of infection and release of virus by the other cells in the culture. No specific SV 40 cytopathogenic effect was observed but it must be pointed out that such an effect could readily have been missed in a few cells.

Induction of appearance of SV 40 virus by prolonged serial propagation of SV 40 tumor cells in vitro: The experiments summarized in Table 3 clearly show that among tens of millions of SV 40 tumor cells, which during serial cultivation *in vitro* over a period of a month or more failed to yield any detectable infectious virus, there ultimately appeared a spontaneous release of minute amounts of SV 40 virus—at

TABLE 3

INDUCTION OF APPEARANCE OF SV 40 VIRUS BY PROLONGED SERIAL PROPAGATION OF SV 40 TUMOR CELLS *in vitro*

A 20th transplant passage tumor (Exp. 1) and a 30th transplant passage tumor (Exp. 2) were used to initiate the cultures by planting 2×10^7 trypsinized cells in 32 ounce bottles 40 ml of Eagle's minimum essential medium + 30% calf serum were used per bottle originally and each time the medium was changed every 2 or 3 days. Incubation was at 37°C. Each pooled fluid harvest was tested in Cercopithecus BS-C-1 cultures—usually a total of 1 ml in 5 tubes. (Positive tests in bold face print)

Exp. no.	Passage no.	Interval between passages	Virus in culture fluid or in frozen and thawed cells on indicated day																	
			2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	1	..												**0/7**[a]								
	2	13	0/7		0/7		0/7		0/5													
	3	9		0/5		0/5		0/5		0/5		0/5										
	4	11	n.t.		n.t.		0/5		0/8		0/5											
	5	10	0/5		0/5		0/5		0/5		**2/5**		0/5		0/5		**1/5**		n.t.	**2/5**		
	6	19	0/5		0/5		0/5		**5/5**		0/5		**4/5**			**5/5**		**5/5**		**5/5**		**5/5**
	7	21	**5/5**		**5/5**			**5/5**		**5/5**		**5/5**		**5/5**	**4/4**[b]							
	8	14	**4/5**		n.t.		**4/5**		**5/5**		**5/5**			**5/5**		**5/5**		**5/5**			**5/5**	
	9	20	**5/5**			**5/5**		**3/3**														
	10	8			0/5		**4/5**		0/5			**3/5**		**5/5**	**4/4**[c]							
	11[d]	13	0/3		0/5		0/5		0/5		0/5		**1/5**		**1/5**							
	12[e]	15	**3/4**[f]	**3/4**		0/4		**1/4**		0/4		0/4		0/5		**5/5**		**3/5**	**1/1**			
	13[g]	18	0/5[f]	**2/5**		0/5		0/4		0/5		**1/5**			0/5	**5/5**	**5/5**[h]					
	14[g]	15	**2/5**		0/5		0/5	0/5		**5/5**[h]												
								2/5[i]														
	15[g]	7	0/5		0/5		0/5		**2/5**	**5/5**	**5/5**[h]											
										2/5[i]												
	16[g]	9	**4/5**		**3/5**																	
2	1	..	0/5[f]	0/5		0/5		0/5		0/5		0/4		0/5		0/5		0/5	0/5	0/5		0/4[e]
	2	18	0/5		0/5		0/5		0/5		0/5											
	3	8	**5/5**		n. t.		**1/4**		**1/4**		**4/5**	**3/4**										

[a] 7×10^5 trypsinized cells frozen and thawed 3 times.
[b] 1.4×10^6 trypsinized cells frozen and thawed 3 times.
[c] 4×10^5 cells and medium frozen and thawed 3 times.
[d] Poor growth.
[e] One 16-ounce bottle was planted because of smaller number of cells (2×10^6) in previous passage.
[f] 1-day fluid containing many unattached cells was tested.
[g] Two 16-ounce bottles planted with about 5×10^6 cells in each.
[h] 5×10^5 cells and medium frozen and thawed 3 times.
[i] 2×10^6 trypsinized cells frozen and thawed 3 times.

Note: The amount of virus recovered was small in all instances, since the SV 40 cytopathogenic effect in the BS-C-1 tubes was not detected until 16–37 days after addition of the test materials, indicating that even fluids which produced a virus effect in 5/5 tubes could be expected to be positive only in a 1:10 dilution.

first irregularly, then with great regularity during three passages over a period of about 50 days, followed by a continuing pattern of irregular release. It should be pointed out here that at each passage approximately $10^{7.3}$ cells from a single bottle were trypsinized, and the washed sedimented cells were planted in two bottles of similar size. Although the 20th transplant passage tumor used for initiating the cultures for Experiment 1 was not tested for virus (the tissue cultures were started for another purpose), the 30th transplant passage tumor used for Experiment 2 was tested with negative results in 20 BS-C-1 tubes (extract from 0.6 gm of tumor).

Absence of multiplication of added SV 40 virus in SV 40 tumor cells in tissue culture: For an adequate interpretation of the data presented in Table 3 it was necessary to know whether SV 40 virus can multiply after addition to tumor cells in tissue culture. If multiplication could occur under our conditions of cultivation, the increased amounts of virus in some of the later passages could be interpreted as being due to superinfection of nonvirus-yielding cells. If, however, there was no multiplication of added virus, the increased amounts of virus in Experiment 1 in Table 3 would have to be interpreted as indicating either release of larger amounts of virus by individual cells or release of virus by a larger number of cells, i.e., induction of production of infectious virus in a higher proportion of the cell population. The data shown in Table 4, an experiment in which about 3 TCD_{50} of SV 40 virus were

TABLE 4

ABSENCE OF MULTIPLICATION OF ADDED SV 40 VIRUS IN 31ST TRANSPLANT PASSAGE SV 40 HAMSTER TUMOR CELLS GROWN IN TISSUE CULTURE

The tumor used for this experiment contained no demonstrable virus when a frozen and thawed extract of 0.6 gm of tumor tissue and 4×10^7 trypsinized viable cells, frozen and thawed 3 x, were tested for virus in a total of 30 Cercopithecus BS-C-1 tissue culture tubes. The tumor tissue cultures were used 8 days after planting of the trypsinized cells at a time when each tube contained a sheet of about $10^{6.3}$ viable cells. $10^{5.5}$ TCD_{50} of SV 40 virus was added to each tube; adsorption for 2 hr at 37°C was followed by 4 washings to remove unadsorbed virus. Each tube contained 2 ml of MEM medium + 30% calf serum which was replaced every 2 days, the fluid medium being saved for virus tests. Incubation at 37°C. Two virus-inoculated and 2 control tubes were frozen every 2 days for virus tests.

Time after adsorption and washing days	Log 10 TCD_{50} of Virus per Tissue Culture Tube in Indicated Category			
	Tubes with added virus		Tubes without added virus	
	Cells and medium frozen and thawed 3 x	Medium only[a]	Cells and medium frozen and thawed 3 x	Medium only
0	4.5	..	0	0
2	4.2	..	0	0
4	3.0	2.5	0	..
6	2.5	2.5	0	0
8	<2.0	1.5	0	..
12	<2.0	..	1.5	..

[a] Pool of fluids from medium change in remaining tubes submitted to further incubation.

added per cell, clearly indicate that the added SV 40 virus did not multiply in the SV 40 tumor cultures. Moreover, it is of interest that among the control tumor cells without added virus one or more cells spontaneously released a minute amount of virus between 8 and 12 days after initiation of the experiment or about 20 days after the tumor cells were planted in tissue culture.

Evidence that most or all SV 40 tumor cells can give rise to progeny capable of occasionally yielding minute amounts of infectious virus: Although the results of the preceding experiments cannot be explained on the basis of "carry-over" of virus with multiplication in susceptible cells within the tumor, *in vivo* or *in vitro*, as has been demonstrated for polyoma tumors of mice, it was necessary to know whether only a small proportion of the SV 40 tumor cells carry noninfectious SV 40 viral genome or whether it is present in most, or all, of the tumor cells. During the course

TABLE 5

EVIDENCE THAT MOST OR ALL SV 40 TUMOR CELLS CAN GIVE RISE TO PROGENY CAPABLE OF OCCASIONALLY YIELDING MINUTE AMOUNTS OF INFECTIOUS SV 40 VIRUS

Source of small numbers of trypsinized cells used for obtaining progeny tested.	Test for Virus in Tumors Resulting From 2 cells: Hamster No.	2 cells: Result	10 cells: Hamster No.	10 cells: Result
Transplant passage 16. Original tumor cells.	1	10/10	1	0/10; 2/10[a]
	2	10/10		
	3	0/10 → 8/10[b]		
Transplant passage 17 tumor. One passage in tissue culture.	4	5/10	2	9/10
			3	4/10
Transplant passage 27 tumor. One passage in tissue culture.			4	18/19
			5	0/20
			6	0/20
			7	0/20
			8	0/20
			9	0/20
			10	0/17
			11	0/13
Transplant passage 20 tumor; 7 serial passages in tissue culture.	5	12/19	12	17/17
	6	9/18	13	17/20
	7	8/20	14	16/20
	8	6/20	15	9/14
	9	6/20	16	7/11
	10	0/20	17	11/19
	11	0/20	18	7/17
	12	0/16	19	0/20
			20	0/20
Total yielding virus.	9/12		11/20	

[a] Metastatic tumor in axillary lymph node measuring 25 × 22 × 12 mm.
[b] Tumor resulting from passage of 20% suspension of "negative" tumor cells from hamster No. 3 into new hamster.

of other experiments we[7] found that SV 40 tumor cells transplanted in tissue culture can produce progressively growing tumors in about 50 per cent of untreated adult hamsters when only two trypsinized cells were inoculated subcutaneously. Accordingly, tumors that had been produced in hamsters inoculated with about two cells or ten cells were harvested 19 to 57 days after they first became palpable, and extracts were tested for virus, in the manner previously described. The results shown in Table 5 indicate that minute or small amounts of virus were recovered from 9 of the 12 tumors, each derived from about two cells, and from 11 of 20 tumors, each of which was derived from about ten cells. It is noteworthy that a tumor which in a single test of about 0.3 gm failed to yield virus could on transplantation of about 0.1 gm of tissue to another hamster give rise to a tumor that yielded a minute amount of virus (see hamster No. 3 under two cells in Table 5). We also observed the reverse that tumors with about 1,000 PFU of virus per gm may on transplantation of 0.1 gm yield tumors without demonstrable virus. Another noteworthy observation was that a tumor developing at the site of inoculation of a small number of cells may yield no virus in the amount tested, while a distant metastatic tumor from the same hamster could yield a trace of virus in a similar quantity of tumor tissue (see hamster No. 1 under ten cells in Table 5). It should also be noted that after seven serial passages in tissue culture not only did eight of nine adult hamsters inoculated with about two cells each develop large tumors, but that five of eight of these tumors yielded minute amounts of infectious SV 40 virus. There can be no question of "carry-over" of virus with the two-cell or ten-cell inocula. It is noteworthy that the frequency of virus-yielding tumors is no less after transplantation

of two cells than after the routine transplantation of about five million cells. The present data can best be explained by the assumption that almost all, and probably all, SV 40 tumor cells transmit to their progeny noninfectious viral genome and that only a rare cell among the millions in the progeny population is spontaneously induced to yield infectious virus.

Summary.—Very small amounts of SV 40 virus were recovered irregularly and only from occasional SV 40 virus induced tumors during the course of 31 serial transplant passages in adult hamsters. Tumors without virus could give rise to tumors with virus and vice versa. The hamsters bearing the transplanted tumors had no neutralizing antibody, and the tumor extracts contained no inhibitors for small amounts of SV 40 virus. In primary cultures, large numbers of tumor cells either failed to release virus or did so in single bursts of minute amounts on rare occasions. Viral antigen was not detected by the fluorescent antibody technique in the nuclei of tumor cells in primary cultures. Prolonged propagation and serial passage of the tumor cells in tissue culture induced the release of larger, though still small, amounts of virus in cultures that were previously virus-free for a month or more. There was no production of infectious virus when large amounts of SV 40 virus were added to *in vitro* cultures of the tumor cells. Nine of 12 tumors produced by transplantation of approximately two cells in adult hamsters yielded minute amounts of virus, indicating that most, and perhaps all, of the tumor cells carried the SV 40 viral genome in a noninfectious state and that maturation of the virus occurred only rarely in an occasional cell. The similarity between these findings and bacterial lysogeny requires further comparative study.

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*A MATHEMATICAL TREATMENT OF THE DISTRIBUTION OF TIDAL VOLUME THROUGHOUT THE LUNG**

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Classical physiology has been mainly concerned thus far with the measurement of "average" values of physical quantities. It is obvious that the manner in which they are distributed with respect to some frame of reference is also of importance.

In the field of respiration, in particular, some problems arise in connection with the distribution of one or several functional characteristics of the pulmonary system. A mathematical analysis leading to the solution of such problems is, therefore, most indicated.

The general principles involved in such problems can be summarized as follows. Let us suppose a system into which an ideal tracer could be introduced according to a certain law in function of time. Let us further assume that such a system is made up of an extremely large number of units possessing certain characteristics (A) upon which the behavior of the tracer will be dependent in each of them. Let us finally postulate that these characteristics are distributed throughout the system according to a given law (B). Then the behavior of the tracer at the output of the system as a whole (C) is uniquely defined by conditions (A) and (B). The problem to be solved here is just the inverse of the preceding one, namely: Condition (C) being given and (A) being appropriately defined in terms of some physical necessity, to infer the law of distribution (B) of the characteristics of the system. As will be seen later, this leads most often to integral equations the solution of which is identified with the distribution law of such characteristics. As an example, we will deal here with the distribution of tidal volume throughout the lung.[1] In this particular case, the behavior of the tracer at the output of the system (C) is defined by its washout function. When the input of the latter into the lungs is made equal to zero, the behavior with time of the tracer (A) in every unit depends upon the particular characteristic of the unit, which in this case is its volumetric variation. Thus, the distribution function (B) to be dealt with here will be that of the tidal volume throughout the lung. For the specific purpose of this study, we will now briefly describe the "ideal" model of the lung.

Preliminary Considerations Concerning the Analytical Model of the Lung.—For the analytical treatment which follows, the lung will first be considered as being subdivided into an infinitely large number of extremely small independent units of equal initial volume v_0, each of them being subjected to different degrees of periodical expansion Δv. Both quantities Δv and v_0 are extremely small, but their dimensionless ratio $\Delta v/v_0$ can take any positive numerical value from zero to infinity. The initial volume v_0 of the units is here an invariant quantity. The size and number of such units need not be known. Moreover, they do not stand for any specific anatomic structure of the lung. The actual lung units can be defined in terms of mere structures[2–4] or in terms of function and structure.[5] The problem is now to

find out, in the first place, how a particular unit would behave and, in the second place, how the variable quantity $\Delta v/v_0$, which we will call the specific tidal volume of these ideal units, is distributed throughout the lung. As will be seen subsequently, this treatment yields a continuous law of distribution of the phenomenon.

Conditions Prevailing in a Particular Lung Unit.—If, in the inspired air, a gas like nitrogen (or, still much better, argon) is suddenly replaced by another gas, then the dilution of the former in any particular one of these lung units and at any time is entirely defined by the differential equation

$$v\frac{dC}{dt} + C\frac{dv}{dt}\sum_{i=0}^{\infty}(-1)^i\Upsilon\left(t - \frac{iT}{2}\right) = 0, \tag{1}$$

where the inspiratory and expiratory phases are compounded in such a way that the conditions in the lung units at any instant are continuously represented from the origin of time to infinity. Here, $C(t)$ is the concentration of the gas in the lung unit at any time t, v is the variable volume of the unit, T is the period of the phenomenon; the symbol capital upsilon Υ under the infinite summation stands for the Heaviside's unit function of the arguments indicated. The solution of the differential equation (1) is given by equation

$$\mathrm{Log}_e\frac{C(t)}{C(O)} = \sum_{i=0}^{\infty}\Upsilon\left(t - \frac{2i+1}{2}\right)\mathrm{Log}_e\frac{v_0}{v_1} + \sum_{i=0}^{\infty}(-1)^i\Upsilon\left(t - \frac{iT}{2}\right)\mathrm{Log}_e\frac{v_0}{v(t)}, \tag{2}$$

where most of the symbols have the same meaning as before, C_0 being the initial concentration of the gas; v_0 and v_1 are minimum and maximum volumes of the lung unit respectively. The graph given in Figure 1 is a schematic representation of what is implied in equations (1) and (2). It can be observed that the rate of dilution of the gas in the lung unit during the inspiratory phase is solely dependent upon the manner its volume v increases with time and that during the expiratory phase the concentration of the gas remains constant. This obviously implies that the phenomenon described here is uniquely dependent upon the purely mechanical motion of the lung units. As will be discussed later, this is not entirely true: other factors such as the diffusion of the gas from the blood as well as gradients of gas concentration in the air phase are also coming into play, although most of the time to a negligible extent.

Output Concentration of a Particular Lung Unit.—After these considerations concerning what is occurring in the lung units themselves, we will now define measurable quantities such as the actual output of the diluted gas during the successive phases of expiration. We first designate by the symbol ξ_m the specific tidal volume of a given unit of order m, so that

$$\xi_m = \left(\frac{\Delta v}{v_0}\right)_m. \tag{3}$$

Then the gas concentration $C(t)$ at the output of a particular lung unit of order m is given by the equation

$$C(\xi_m, t) = \frac{C(\xi_m, 0)}{(1+\xi_m)]^n}\left[\frac{1}{2} - \frac{2}{\pi}\sum_{0}^{\infty}\frac{\sin\left(\frac{2n+1}{T}2\pi t + \theta_m\right)}{2n+1}\right], \tag{4}$$

$$(n = 0, 1, 2, 3, \ldots)\ (m = 0, 1, 2, 3, \ldots)$$

where n is the breath order and θ_m stands for the phase angle of the periodical phenomenon; the whole term in brackets is derived from the Fourier expansion of unity. The curve corresponding to equation (4) is schematically given by the discontinuous graph of Figure 2. In the first part of this paper, we shall not be concerned with

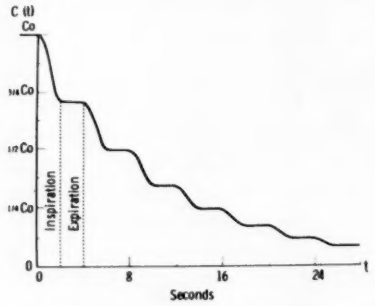

FIG. 1.—Schematic graphs describing the gas concentration with time in a particular lung unit m according to equation (2).

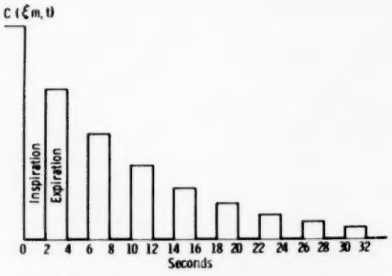

FIG. 2.—Graphic description of output concentration in a particular lung unit at any time as defined by equation (4).

expression (4), such as it is given here, but with its envelope tangential to the definite instants corresponding to the end of every expiratory phase. The specific curve corresponding to each individual breath either at the output of the units or after they have been summed will be considered later. When θ_m is constant from unit to unit, then equation (4) can readily be replaced by the equation

$$C(\xi_m, n) = C(\xi_m, 0)\left(\frac{1}{1 + \xi_m}\right)^n \tag{5}$$

representing the end tidal concentration $C(\xi_m, n)$ of the gas for every value of n and for every lung unit of order m.

Summation of the Output Concentration of All Lung Units.—It is clear, from the definitions given before, that if all these lung units are taken together the average concentration $\bar{C}(n)$ of the gas at the output of the system will be defined by the equation

$$\bar{C}(n) = \frac{v_0}{V_T}\sum_0^M \frac{C(\xi_m, 0)\xi_m}{(1 + \xi_m)^n}, \tag{6}$$

where $C(\xi_m, 0)$ stands for the initial concentration in a particular unit of order m. This is considered here as being different from unit to unit. V_T stands for the overall tidal volume of the system and M is the total number of units involved.

Passage from the Summation of All Units to an Integral Equation.—If now we allow the units in this system to become infinitely small, then the number M becomes infinitely large. We are thus entitled to consider the discrete variable ξ_m as a continuous one ξ in which, from now on, we drop the subscript m. Then, taking into account that a distribution function $f(\xi)$ of these continuous values of ξ necessarily exists, the summation (6) is easily transformed into the definite integral

$$\bar{C}(n) = \frac{v_0}{V_T} \int_{\xi_1}^{\xi_2} \frac{C(\xi, 0)\xi f(\xi)}{(1 + \xi)^n} d\xi, \tag{7}$$

which, in the manner so written, constitutes a linear integral equation of the first kind for the determination of the distribution function $f(\xi)$ once the function $\bar{C}(n)$ is algebraically known.

The Distribution Functions of Specific Tidal Volume.—Obviously the very existence of the distribution function $f(\xi)$ requires the fulfilment of some conditions, namely:

(*a*) Function $f(\xi)$ and the independent variable ξ itself can have only positive numerical values, those of the former existing only for $\xi_1 \leq \xi \leq \xi_2$. Everywhere else $f(\xi)$ has to be equal to zero.

(*b*) The over-all tidal volume of the system is defined by the integral

$$V_T = v_0 \int_{\xi_1}^{\xi_2} \xi f(\xi) d\xi, \tag{8}$$

which is taken between the limits ξ_1, and ξ_2.

(*c*) The minimum end expiratory volume of the system is defined by the definite integral,

$$V = v_0 \int_{\xi_1}^{\xi_2} f(\xi) d\xi, \tag{9}$$

taken between the same limits as (8).

(*d*) The ratio $\bar{\xi}$ of integral (8) over integral (9), as given by the equality,

$$\bar{\xi} = \int_{\xi_1}^{\xi_2} \xi f(\xi) d\xi / \int_{\xi_1}^{\xi_2} f(\xi) d\xi, \tag{10}$$

must equal that of the over-all tidal volume over the minimum end expiratory lung volume.

Transformations Reducing the Integral Equation to a Laplace's Infinite Integral.—With these conditions fulfilled, we will deal at present with the actual determination of $f(\xi)$. First of all, it is useful to introduce here an auxiliary function $\phi(\xi)$ as defined by the equation

$$\phi(\xi) = \frac{C(\xi, 0)f(\xi)}{\bar{C}(0)}. \tag{11}$$

Function $\phi(\xi)$ is the actual one to be determined and will be identical with function $f(\xi)$ when and only when the initial concentration of the gas $C(\xi, 0)$ is the same for all the lung units and consequently the same as the one $\bar{C}(0)$ of the washout function. Otherwise, what is actually measured is the distribution of the quantity defined by (11). This point will be, however, of great importance for some applications of the theory given later. Introducing (11) into (7), one gets the new integral equation

$$\bar{C}(n) = \frac{v_0 C_0}{V_T} \int_{\xi_1}^{\xi_2} \frac{\xi \phi(\xi)}{(1 + \xi)^n} d\xi, \tag{12}$$

the initial value $\bar{C}(0)$ of the gas concentration at the output of the system being defined now by the expression

$$\bar{C}(0) = \frac{v_0}{V_T} \int_{\xi_1}^{\xi_2} C(\xi, 0)\xi f(\xi) d\xi. \tag{13}$$

The integral equation (12) contains a nonsymmetrical kernel involving the discrete variable n. We make the kernel symmetrical by changing the variable of integration ξ into the auxiliary variable u through the logarithmic transformation of the form

$$\mathrm{Log}_e \frac{1+\xi}{1+\xi_1} = u. \tag{14}$$

With this transformation, the integral equation (12) becomes the new integral

$$\frac{v_0 C_0}{V_T(1+\xi_1)^{n-1}} \int_0^\infty h(u)e^{-nu}du = \bar{C}(n), \tag{15}$$

where ξ_2 has been allowed to become infinite in (12) and where $h(u)$ is the new function derived from the preceding transformation (14).

Taking now the continuous variable p instead of the discrete one n and rearranging the terms in (15), the latter equation becomes

$$\int_0^\infty h(u)e^{-pu}du = \Omega(p), \tag{16}$$

where

$$\Omega(p) = \frac{(1+\xi_1)^{p-1}V_T}{v_0 C_0}\bar{C}(p). \tag{16a}$$

The integral term in equation (16) is none other than the classical Laplace infinite integral in which function $h(u)$ can be determined when $\Omega(n)$, and consequently when $\bar{C}(n)$, is algebraically known.

By making use now of the Mellin-Fourier or Bromwich-Wagner theorem, as expressed by the inversion formula

$$h(u) = \frac{1}{2\pi j}\int_{c-j\infty}^{c+j\infty} \Omega(p)e^{up}dp, \tag{17}$$

in which the integration has to be performed over the complex plane, it is possible to infer function $h(u)$ and consequently the required function $\phi(\xi)$.

Explicit Formulation of the Washout Function.—The final important step now is that of expressing $\bar{C}(n)$, and consequently $\Omega(p)$, as defined by (16*a*) by an appropriate formulation. To this end, we have made it possible in four different ways of which only one will be described in the following. The washout function $\bar{C}(n)$ is defined by the general formula

$$\bar{C}(n) = H(n) + F(nT), \tag{18}$$

where F in the second member stands for a continuously decreasing function of time of very small amplitude and diminishing extremely slowly. This component takes its origin almost entirely from the tissues via the blood supply through the lung units. It is not entirely so, however, in cases with pathological changes in the lungs characteristic of emphysema as will be seen later. The fact that one has to deal with a discontinuous determination $\bar{C}(n)$ (end tidal gas concentration) clearly explains why, instead of $F(t)$, one has to make use of $F(nT)$. The condition defined here is expressed by the equation

$$F(nT) = J_0 e^{-bnT} \tag{18a}$$

where J_0 and b, in normal individuals at least, are just of the order of magnitude expected from the ventilation and perfusion of the lungs. Having subtracted $F(nT)$ from $\bar{C}(n)$, the required function $H(n)$ is now numerically known for every breath.

The function $H(n)$ is expressed by the equation

$$H(n) = \frac{H(0)e^{-sn}}{(1 + an)^z}, \tag{19}$$

which fits almost exactly the experimental washout data.

Parametric Solution of the Washout Function.—To determine the numerical value of the parameters a, z, and s in equation (19) we proceed as follows. On a washout curve such as the one represented in Figure 3, take a value of n at which $H(n)$ is a

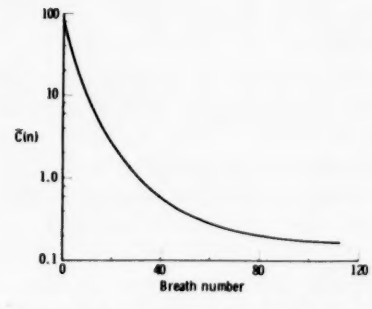

FIG. 3.—Nitrogen washout curve from a normal individual to which equations (18), (18a), and (19) apply.

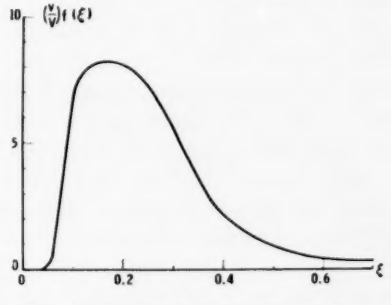

FIG. 4.—Graph of the distribution law of tidal volume as defined by equation (32) and derived from equation (19) and the graph of Figure 3.

very small fraction of H_0. This value of $H(n)$ should be of the order of 0.2 per cent of H_0. Then, designating by H_4 this value of $H(n)$ and by $4n_1$ the corresponding value of n, take now the values H_2 and H_1 of $H(n)$ for values of n equal to $2n_1$ and n_1 respectively. One can then form the three simultaneous equations

$$H_1 x = \frac{H_0}{(1 + \omega)^z}, \tag{20}$$

$$H_2 x^2 = \frac{H_0}{(1 + 2\omega)^z}, \tag{21}$$

$$H_4 x^4 = \frac{H_0}{(1 + 4\omega)^z}, \tag{22}$$

in which x and ω have the definition

$$x = e^{sn_1}, \tag{23}$$

$$\omega = an_1 \tag{24}$$

These are transcendental equations which cannot be solved in closed form; but from (20), (21), and (22), one obtains the parametric equation

$$\frac{\text{Log}_e H_0H_4/H_2^2}{\text{Log}_e H_0H_2/H_1^2} = \frac{2\ \text{Log}_e(1+2\omega) - \text{Log}_e(1+4\omega)}{2\ \text{Log}_e(1+\omega) - \text{Log}_e(1+2\omega)} = \text{R}(\omega), \tag{25}$$

which is easily solved by the use of numerical tables and appropriate graphs constructed here for the purpose of numerical computations. Knowing the value of ω, it is easy to evaluate a, z, and s through the equations

$$a = \omega/n_1, \tag{26}$$

$$e^{sn_1} = \frac{H_0/H_1}{(1+\omega)^z}, \tag{27}$$

$$z = \frac{\text{Log}_e H_0H_2/H_1^2}{2\ \text{Log}_e(1+\omega) - \text{Log}_e(1+2\omega)} = \frac{\text{Log}_e H_0H_4/H_2^2}{2\ \text{Log}_e(1+2\omega) - \text{Log}_e(1+4\omega)}, \tag{28}$$

$$s = \frac{1}{n_1}\left(\text{Log}_e H_0/H_1 - z\text{Log}_e(1+\omega)\right). \tag{29}$$

Now to obtain $h(u)$ and consequently $\phi(\xi)$, one has to introduce $H(n)$ from (19) into the Mellin-Fourier inversion formula (17), thus obtaining $h(u)$ directly and then $\phi(\xi)$ from the logarithmic transformation (14).

Explicit Form of the Distribution Function of Tidal Volume throughout the Lung.— When the preceding methods of analysis are used, the final distribution function $f(\xi)$ is given by the expression

$$\frac{v_0}{V_T}\,\xi f(\xi) = \mathfrak{B}\,\frac{\left(\text{Log}_e\dfrac{1+\xi}{1+\xi_1}\right)^{z-1}}{(1+\xi)^{\alpha}}, \tag{30}$$

in which, Γ being the gamma function, the parameters involved have the meanings defined by the equations

$$\left.\begin{aligned} \alpha &= \frac{1}{a} + 1 \\ \text{Log}_e(1+\xi_1) &= s \end{aligned}\right\}, \tag{30a}$$

$$\mathfrak{B} = \mathfrak{B}(\alpha, z, \xi_1) = \frac{(\alpha-1)^z(1+\xi_1)^{\alpha-1}}{\Gamma(z)}. \tag{31}$$

Another way of expressing the distribution function is given by the equation

$$\frac{v_0}{V} f(\xi) = \mathfrak{A}\,\frac{\left(\text{Log}_e\dfrac{1+\xi}{1+\xi_1}\right)^{z-1}}{\xi(1+\xi)^{\alpha}}, \tag{32}$$

in which the factor $\mathfrak{A}$ is expressed by equation

$$\mathfrak{A} = \mathfrak{A}(\alpha, z, \xi_1) = \frac{(1+\xi_1)^{\alpha}}{\Gamma(z)\displaystyle\sum_{i=1}^{\infty}\frac{1}{(1+\xi_1)^i\alpha^{z+i}}} \qquad (\xi_1 \geq 0). \tag{33}$$

Equations (31) and (33) define normalizing factors for $\mathfrak{B}$ and $\mathfrak{A}$ respectively. Here, $f(\xi)$ instead of $\phi(\xi)$ is taken because $\bar{C}(0)$ changes but very little around a fixed value of 81 per cent,[5] so that in equation (11) one has $C(\xi, 0) \approx \bar{C}(0)$. Thus, while equations (30) and (32) are in keeping with equations (8) and (9), division of equation (30) by (32) furnishes the average value $\bar{\xi}$ of ξ required by (10), so that

$$\bar{\xi} = \frac{\mathfrak{A}}{\mathfrak{B}} = \frac{(1 + \xi_1)}{(\alpha - 1)^z \sum_1^\infty \frac{1}{(1 + \xi_1)^i \alpha^{i+z}}}, \tag{34}$$

which is uniquely defined now by the parameters involved in the law of distribution.

Example of Distribution Function in a Normal Lung.—An example of the distribution function in a normal case as given by equation (30) is represented by the graph of Figure 4. As can be seen in this case, where the tidal volume was of 1,100 cc, there exist all kinds of numerical values of the amount of ventilation from $\xi = 0.04$ to infinity, but those units having a specific tidal volume of the order of 0.18 are in the greatest number and their number becomes smaller and smaller as ξ increases indefinitely. Formulas (30) and (32) are dimensionless and have been normalized in such a way that their integral value under the curve is equal to unity.

Conclusions.—The method of analysis described in this paper yields a continuous law of distribution of tidal volume throughout the lung. Application of the preceding analytical methods to experimental washout data from normal and pathological individuals is now under study.

The author is indebted to Doctors Briscoe and Cumming for having provided him with nitrogen washout data from which the graph of Figure 3 has been constructed.

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*THE HIGH-FREQUENCY SENSITIVITY OF THE GUINEA PIG EAR**

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The investigation of the hearing of animals in the ultrasonic range has been facilitated by the development of improved apparatus for the production and measurement of high tones. In this investigation it has become obvious that for many animals the reception of high tones constitutes a very important part of their sensory capability. The most striking evidence here is the use of the uppermost frequen-

cies by bats in the process of echolocation,[1] and there are indications that something similar occurs in porpoises[2] and perhaps also in rats,[3] shrews,[4] and certain species of birds.[5]

What further use these animals make of the upper frequencies is yet unknown, though the high degree of sensitivity exhibited by the ones in which acuity tests have been made is at least suggestive that the high tones play a significant part in such processes as communication, location of prey, and avoidance of enemies.

So far the systematic study of sensitivity to the very high tones has been carried out only in cats,[6] marmosets,[7] and bats,[8] though a few measurements have been made in dogs,[9] rats,[10] mice,[11] porpoises,[12] and other experimental animals.[13]

The present experiments are concerned with the high-frequency sensitivity of guinea pigs as revealed in the cochlear potentials. This animal has long held a favored position as the subject of various kinds of investigations of hearing, and has been used most extensively in studies of Preyer's reflex and stimulation deafness. Measurements have been made of its auditory sensitivity by conditioned response methods and by means of the cochlear potentials, but only within a limited range of frequencies. Horton's determinations[14] of sensitivity by means of a conditioned respiratory response extended from 64 to 8,192 cycles, and most measurements of cochlear potentials in this ear have not gone beyond 15,000 cycles, though potentials have been reported for uncalibrated tones up to 35,000 cycles[15] and 57,500 cycles.

Method and Procedure.—The observations were made on a series of 14 guinea pigs which were deeply anesthetized with 20 per cent ethyl carbamate (Urethane) in mammalian Ringer's solution. During stimulation with tones, the cochlear potentials were recorded from the round window membrane with either a platinum foil or a silver bead electrode, and with an indifferent electrode in inactive tissue near by.

Tones over the range from 50,000 to 100,000 cycles were produced by means of electrostrictive rods of barium titanate, and the lower tones, down to 100 cycles, with a moving-coil loudspeaker (Western Electric Type 555), and were led to the external auditory meatus through a tube. Measurements of sound pressure at the entrance to the meatus were made with a condenser microphone and probe system calibrated over the complete range up to 100,000 cycles. Usually 22 tones were used to cover the range from 100 to 100,000 cycles, and were applied at the sound pressures necessary to produce an arbitrary level of response.

Representative results are shown in Figure 1. The curve represents a typical ear, and the other points show the responses of two other ears within the range that we regard as normal. These data represent sensitivity functions and show in decibels relative to 1 dyne per sq cm the sound pressure required to produce a standard response of 1 microvolt. The three ears are closely similar in the low frequencies, but show moderate variations in the high frequencies. The form as indicated for the tones up to 15,000 cycles is much the same as reported previously for this range. There is a maximum of sensitivity in the region of 500 to 700 cycles, which is probably a region of mechanical resonance for the guinea pig ear. From here upward the sensitivity shows some variations but is fairly well maintained until the region of 40,000 to 55,000 cycles is reached, after which the sensitivity rapidly declines.

In the uppermost frequencies, from 70,000 to 100,000 cycles, there were consider-

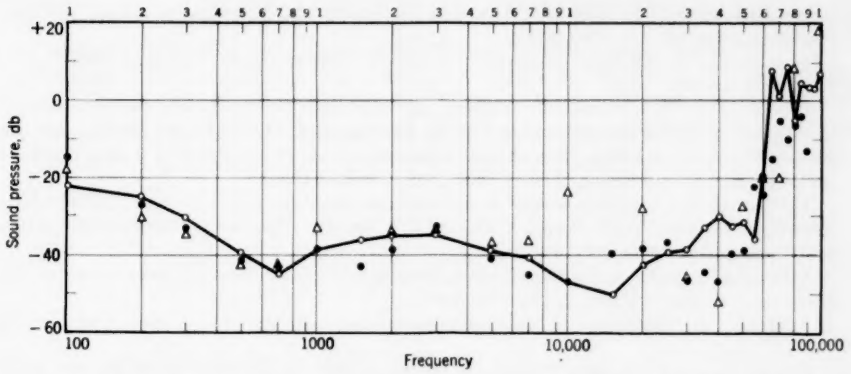

FIG. 1.—Sensitivity functions for three guinea pig ears, as indicated by the different kinds of points. Each point represents the sound pressure, in decibels relative to 1 dyne per sq cm, required to produce a potential of 1 microvolt at the indicated frequency.

able differences among the ears of our group. In some animals the decline in sensitivity continued all the way to 100,000 cycles, but in others it leveled off, or even reversed its direction, in the region of 80,000 to 100,000 cycles. In a number of animals there was a slight maximum at 10,000 or 15,000 cycles, and sometimes a more prominent maximum in the region of 30,000 to 45,000 cycles, but these features were not regular enough in appearance to suggest any characteristic resonances in the auditory system. They evidently represent individual variations in mechanical or other properties.

It would be of interest to compare the sensitivity functions obtained in these experiments with behavioral data on the guinea pig, but unfortunately, as already mentioned, such data are not available for the range above 8,192 cycles. At this time we can only offer a prediction as to the character of the behavioral acuity function at the upper end of the scale based on the cochlear potential results.

The evidence on cats and marmosets, in which both the cochlear potential and behavioral curves have been extended to the upper frequencies, shows that the acuity fails to be sustained to the highest frequencies for which cochlear potentials are measurable. This difference probably arises from two circumstances, a sparsity of nerve fibers in the extreme basal region of the cochlea where the highest tones have their main effect, and a reduced excitability of the nerve fibers by the cochlear potentials at these extreme frequencies. Accordingly, we expect that the behavioral acuity of the guinea pig ear will be well maintained up to a frequency of about 55,000 cycles, and thereafter will rapidly fail.

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† Postdoctoral fellow in a training program in Sensory Psychology, supported by the National Institutes of Health (NINDB).

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*BIOSYNTHESIS OF VALINE AND ISOLEUCINE, VI. ENZYME REPRESSION IN SALMONELLA**

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Studies on *Escherichia coli* K-12[1] have shown that valine inhibits the growth of this organism as a result of a feedback control exerted by valine on the first enzyme (α-hydroxy-β-ketoacid-forming enzyme) in the common pathway (Fig. 1) for the biosynthesis of valine and isoleucine. It has been shown also that the *E. coli* strain W, whose growth is not affected by valine, and a valine-resistant mutant of *E. coli*, K-12, possess α-hydroxy-β-ketoacid-forming systems that are less sensitive to valine, and, as a consequence, are not subjected to the feedback-control mechanism described for *E. coli* K-12.[2] More recently, Freundlich *et al.*[3] have presented additional data indicating that in the *E. coli* strain W and *Salmonella typhimurium* the biosynthesis of valine and isoleucine is controlled by means of an enzyme repression mechanism exerted by valine, isoleucine, and leucine. All three

amino acids are required to be present together. There is a direct relationship between the biosynthesis of valine and isoleucine (see Fig. 1) and the biosynthesis of leucine. The valine precursor, α-ketoisovalerate (α-ketovaline), has been shown to be a precursor of leucine.[4] Therefore, it appears that the end products of *both* pathways are required for the repression of the pathway controlling the biosynthesis of valine and isoleucine. This type of repression has been termed "multi-valent" by the authors.

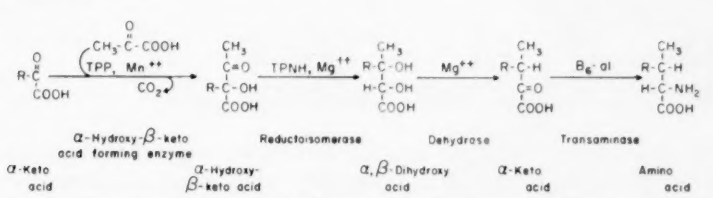

FIG. 1.—Reactions involved in the biosynthesis of valine and isoleucine. R, CH_3 for valine biosynthesis, CH_2CH_3 for isoleucine biosynthesis.

The present report concerns a study carried out in this laboratory on the effect that valine, isoleucine, and leucine have on the four enzymes that constitute the common pathway for the biosynthesis of valine and isoleucine in wild-type *S. typhimurium* under various experimental conditions.

Methods.—The organism used in this study was wild-type *S. typhimurium* strain LT-2. The minimal medium of Davis[5] or Difco nutrient broth was used to grow the bacteria. The cells were grown under forced aeration at 37°. Growth attained was determined by measuring turbidity as optical density at a wavelength of 550 mμ with a Bausch and Lomb Colorimeter.

Cells were harvested in a Lourdes refrigerated centrifuge equipped with a continuous flow rotor, then washed with cold isotonic saline. The cell suspension was centrifuged at 12,000 $\times$ *g* for 10 min in a Lourdes centrifuge, then suspended in cold 0.1 *M* KPO_4 buffer, pH 7.5, and treated in a 20-Kc Bronwill Biosonik for 10 mins. The resultant suspension was centrifuged for 30 min at 35,000 rpm in a Spinco Model L ultra centrifuge, and the supernatant fraction diluted with cold 0.1 *M* KPO_4 buffer, pH 7.5, to a protein concentration of 10 mg per ml. Protein was determined by the method of Lowry *et al.*[6] Conditions of growth and treatment of the cells for the individual experiments are given with the tables and figures.

Enzymatic assays: The assay for the α-hydroxy-β-ketoacid-forming enzyme (α-acetolactate-forming enzyme) has been described by Radhakrishnan and Snell[7] for *E. coli*. This enzyme in *Salmonella*, like that in *Neurospora crassa*,[7] shows one pH optimum between 6.0 and 6.5. The substrate used in the assays described here was sodium pyruvate. The reductoisomerase was assayed according to the method published by Armstrong and Wagner.[8] The substrate in the assays was α-aceto-α-hydroxybutyrate which was prepared according to the method of Krampitz.[9] Dehydrase activity was determined by the method described by Wagner, Bergquist, and Karp,[10] and the substrate (α, β-dihydroxy-β-methylvalerate or α, β-dihydroxyisovalerate) used is indicated in the individual tables and figures. These substrates were prepared according to the method of Sjolander *et al.*[11] The dihydroxy acid of isovalerate is 1.5 to 2-fold as active as that of β-methylvalerate in

TABLE 1

EFFECT OF VALINE, ISOLEUCINE, AND GLYCINE ON *in vitro* ENZYMATIC ACTIVITIES

Addition to assay	Specific Activity (μ moles per hr per mg*)					
	Acetolactate-forming enzyme	Reducto-isomerase	Dehydrase DHV†	Dehydrase DHI	Dehydrase DHV	Trans-aminase
None	0.25	8.9	2.2	1.4	6.1	1.1
L-valine (0.1 mMole)	0.24	8.5	2.2	1.2	3.0	0.2
L-isoleucine (0.05 mMole)	0.24	9.0	2.1	1.2	3.7	0.3
L-valine (0.1 mMole) and *L*-isoleucine (0.05 mMole)	0.25	8.6	2.2	1.4	3.0	0.0
Glycine (0.1 mMole)	0.24	9.0	2.2	1.2	3.7	1.1

* See *Methods* for references and details concerning the enzymatic assays.
† DHV = α,β-dihydroxyisovalerate, DHI = α,β-dihydroxy-β-methylvalerate.

the dehydrase assay. The transaminase activity was carried out according to the procedure published by Seecof and Wagner,[12] using α-ketoisovalerate as the substrate. This substrate was prepared according to the method of Meister.[13] Threonine deaminase activity was determined by the method of Yanofsky and Reissig.[14] One mg of protein was used in all assays except in the case of the reductoisomerase assay in which 0.2 mg of protein was used. Enzymatic activities are recorded as specific activity, μmoles of substrate converted per hr per mg of protein.

Results.—The effects of the addition of valine and isoleucine to the *in vitro* assays of the four enzymes of the pathway are given in Table 1. *L*-valine and *L*-isoleucine, alone or in combination, have no effect on the activities of the α-acetolactate-forming and reductoisomerase enzymes. The same is true of the dehydrase activity, unless the activity present in the cell-free extract is high (specific activity: 5–6), then a reduction is noted. However, in this case, the addition of glycine to the assay results in a comparable loss. Thus, the observed decline in activity appears to be due to the instability of the labile dehydrase in the presence of the addenda. An inhibition is noted when valine and/or isoleucine are added to the transaminase assay; this loss of activity is considered to be the result of product inhibition. The *in vitro* data, therefore, provide no evidence of a feedback inhibition of the enzymes involved in the biosynthesis of valine and isoleucine.

To demonstrate enzyme repression, the enzymes of the pathway were assayed after cells were first grown in nutrient broth and then after they were suspended in minimal medium. As seen in Table 2 both the reductoisomerase and the dehydrase are repressed in nutrient broth and derepressed in minimal medium. Attempts to prevent the release of repression by the addition of valine and isoleucine to the medium (Exps. I and II of Table 2) are ineffective unless high levels (10 mM) of isoleucine are present and then the release of the reductoisomerase is affected. At times, this effect appears to be antagonized by the concurrent presence of valine (Exp. II); this type of an antagonism is not considered unusual.[15] With an isoleucine + valine + leucine supplement, the release of repression of the reductoisomerase and, to a lesser extent, the dehydrase is inhibited (Exp. III). The increase in α-acetolactate-forming enzyme activity is also inhibited by this supplement. However, we were unable to detect a consistent inhibition of transaminase activity. In those experiments in which inhibition may have occurred, the difference between the derepressed activity and that in the presence of the valine + isoleucine + leucine supplement was about 2-fold or less.

TABLE 2

EFFECT OF VALINE AND ISOLEUCINE ON THE RELEASE OF ENZYME REPRESSION

Salmonella cells were harvested after 15 hr growth in nutrient broth. A cell-free extract was prepared from an aliquot of these cells and is labeled "repressed." Aliquots of the remaining cells were suspended in minimal medium supplemented as indicated below and allowed to incubate 2.5 hr. The cells were then harvested, and cell-free preparations were made. See *Methods* for references and details concerning the enzymatic assays. α,β-Dihydroxyisovalerate was used as the substrate for the dehydrase assay in Exps. I and III, and α,β-dihydroxy-β-methylvalerate was used in Exp. II.

Supplement during release of enzyme repression	Specific Activity (μ moles/hr/mg)			
	Acetolactate-forming enzyme	Reducto-isomerase	Dehydrase	Transaminase
Experiment I:				
"Repressed"	0.34	0.8	0.4	1.0
None	0.57	11.6	4.3	1.6
L-valine (2 mM)	0.55	8.4	4.5	1.6
L-isoleucine (2 mM)	0.49	8.2	4.4	1.6
L-valine and *L*-isoleucine (2 mM each)	0.49	8.2	4.3	2.0
Experiment II:				
"Repressed"	0.25	0.8	0.2	1.0
None	0.32	13.6	2.3	2.0
L-valine (10 mM)	0.17	14.2	4.4	2.0
L-isoleucine (10 mM)	0.30	3.0	2.0	1.6
L-valine and *L*-isoleucine (10 mM each)	0.30	7.5	2.0	2.4
Experiment III:				
"Repressed"	0.28	1.5	0.9	1.0
None	0.68	12.4	6.9	2.0
L-isoleucine (10 mM)	0.48	6.7	5.4	2.0
L-isoleucine and *L*-valine (10 mM ea)	0.40	6.3	5.0	2.5
L-isoleucine, *L*-valine and *L*-leucine (10 mM each)	0.27	2.3	2.2	2.0

The patterns of enzyme activity obtained from cells grown in minimal medium supplemented with valine show a depression of the reductoisomerase and the dehydrase (Fig. 2). The severest declines in these two activities, however, are temporary. The specific activity of the α-acetolactate-forming enzyme increases as growth commences, then declines, followed by another increase. Subsequent investigations have shown that the latter increase is the exception rather than the rule; after an initial increase the activity returns to a level comparable to that of the base-line values. Of the four enzymes, the transaminase displays the least amount of fluctuation.

Figure 3 presents a sample of the results obtained when the same experiment described for Figure 2 was carried out with two changes in procedure: (1) cells were suspended in minimal medium containing *L*-isoleucine in addition to *L*-valine, and (2) theonine deaminase activity was included in the survey of enzymatic activities. The α-acetolactate-forming enzyme and the threonine deaminase display patterns of activity similar to each other, as well as to that seen for the α-acetolactate-forming enzyme of Figure 2. Although the activities of the reductoisomerase, dehydrase and transaminase are not included in Figure 3, it should be stated that the patterns of activity noted for these enzymes are comparable to those depicted in Figure 2. However, when isoleucine and valine are added to the medium, the decrease in dehydrase activity is not as marked as when valine alone is the supplement.

When cells are grown in minimal medium supplemented with leucine in addition to isoleucine and valine (Table 3), no significant enzyme repression is noted. Trans-

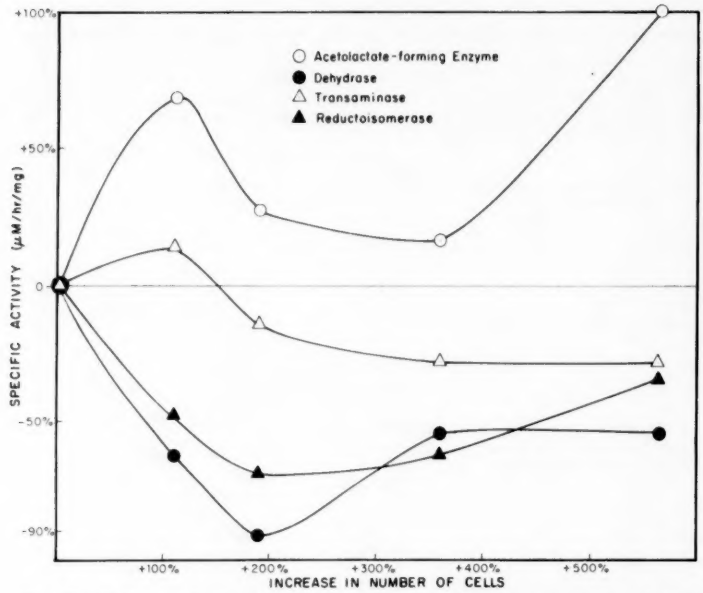

FIG. 2.—Patterns of enzyme activities of *Salmonella* when grown in minimal medium supplemented with valine. Cells were harvested during the late logarithmic phase of growth in minimal medium, and a cell-free extract was prepared from an aliquot of these cells. The enzymatic activities determined in this preparation serve as the base-line values (0%) in the above figure. The remaining cells were suspended in minimal medium supplemented with 2 mM *L*-valine and incubated at 37°. Aliquots of cells were removed after various increments of growth, and cell-free preparations were made. See *Methods* for references and details concerning the enzymatic assays. α,β-Dihydroxy-isovalerate was used as the substrate in the dehydrase assays. The specific activities for the baseline values (0%) are 0.19, 11.3, 6.1, and 1.4 for the α-acetolactate-forming enzyme, reductoisomerase, dehydrase and transaminase activities, respectively.

fer of the cells from a supplemented to an unsupplemented medium results in an increase in enzyme activities (similar to the release-of-repression values obtained in Table 2). Growth studies utilizing supplemented media showed that exogenously supplied valine exerts an effect on both the reductoisomerase and the dehydrase. However, a true repression has never been observed using this experimental ap-

TABLE 3

ENZYME ACTIVITIES OF *Salmonella* GROWN IN MINIMAL MEDIUM WITH AND WITHOUT AN ISOLEUCINE, VALINE, AND LEUCINE SUPPLEMENT

Salmonella cells were harvested during the late logarithmic phase of growth in minimal medium. A cell-free extract was prepared from an aliquot of the cells (labeled: None-I), and the remaining cells were suspended in minimal medium supplemented with 3 mM each of *L*-isoleucine, *L*-valine, and *L*-leucine and allowed to grow until an increase of 300% in the number of cells had been attained. The cells were then harvested, and an aliquot of these cells were used to prepare a cell-free extract (labeled: iso, val, leu). The remaining cells were suspended in minimal medium and allowed to grow. The cells were harvested after 2½ hr and a third cell-free extract was prepared (labeled: None-II). See *Methods* for references and details concerning the enzymatic activities. α,β-Dihydroxy-β-methylvalerate was used as the substrate for the dehydrase assay.

Supplement	Specific Activity (μ moles/hr/mg)			
	Acetolactate-forming enzyme	Reducto-isomerase	Dehydrase	Transaminase
None-I	0.37	10.8	2.0	1.0
L-isoleucine, *L*-valine, and *L*-leucine, (3 mM each)	0.36	7.2	2.5	0.6
None-II	0.67	16.1	5.1	1.1

proach. Neither isoleucine nor leucine appears to enhance the effect of valine, and occasionally even valine (alone or in combination with isoleucine or leucine) has no significant effect on the enzyme activities, e.g., Table 3. Growth of wild-type *Salmonella* in liquid or on solid media is not inhibited when the various combinations of valine, isoleucine, and leucine (in amounts up to 1 mg of the *L*-form per ml) are present in the medium.

Discussion.—The data reported here for *Salmonella* show that valine does not inhibit growth nor does it inhibit the *in vitro* activity of the α-acetolactate-forming enzyme. Thus, the feedback-control mechanism described for *E. coli* K-12[2] is not found in the LT-2 strain of *Salmonella typhimurium*.

An enzyme repression involving the reductoisomerase and the dehydrase has been observed (Table 2). As seen in Experiment III of that table, three amino acids (valine, isoleucine, and leucine) are required to affect significantly the release of the reductoisomerase and, to a lesser extent, the dehydrase. Moreover, this supplement exerts an effect on the α-acetolactate-forming enzyme, an activity not directly involved in the observed repression. Isoleucine has also been shown to affect the release of repression of the reductoisomerase (Exp. II of Table 2). The results of attempts to repress the activities of wild-type *Salmonella* grown in supplemented minimal media have shown that although no true repression was obtained, valine does depress the reductoisomerase and dehydrase activities (an effect not enhanced by the concurrent presence of isoleucine and leucine).

In view of the high levels of exogenous amino acids required to obtain the effects noted in this study, there is the possibility that the amino acids are not concentrated by the cells in the quantities required for total repression. Such a permeable factor is considered below.

A comparison of the data obtained in this study with those presented by Freundlich *et al.*[3] shows what appears to be discrepancies, but the following considerations should be made.

In the present study analyses of the four enzymes that constitute the isoleucine-valine pathway in wild-type *Salmonella* were undertaken; whereas in the study by Freundlich *et al.*[3] analyses of three enzymes (one from each of the following pathways: isoleucine, isoleucine and valine, and leucine) were carried out utilizing auxotrophic strains of *Salmonella*. Common ground for comparison are the results obtained on the dehydrase of the isoleucine-valine pathway and, to a lesser extent, the threonine deaminase.

The reductoisomerase-deficient strains (*ilva A*) utilized by Freundlich *et al.*[3] have been shown by Wagner and Bergquist[16] to possess very high dehydrase activity, as compared to the levels observed in wild type. The high specific activities are associated with the "derepressed" state occurring in the mutant strains. In comparing the results of the two studies, the repression of high levels of dehydrase activity by isoleucine, valine, and leucine results in specific activities (2.6–6.0) that are routinely observed in the wild-type strain. Thus, the studies presented in this report are mainly concerned with levels of activity that are considered maximally repressed in the mutant strains. The results show, therefore, that whereas the "derepressed" systems are sensitive to repression, the dehydrase activity routinely observed in the wild-type strain is not. The isoleucine-valine-leucine effect noted in this study serves as an example for, although the data compare fav-

orably with those obtained by Freundlich *et al.*,[3] *viz.* repression of "derepression" (Table 2), this supplement is *not* effective in repressing the levels of enzymatic activities routinely observed in wild-type *Salmonella* grown in minimal medium (Table 3). These observations are interpreted to mean that although repression does occur in the mutant strains, it is limited, since, in the repressed state, levels of activity capable of supporting wild-type growth are still maintained by the cell. As mentioned previously, a permeability factor may represent a mechanism by which the synthesis of isoleucine and valine are protected from complete repression (or perhaps the control mechanism is "adapted" to the levels of amino acids concentrated by the cell).

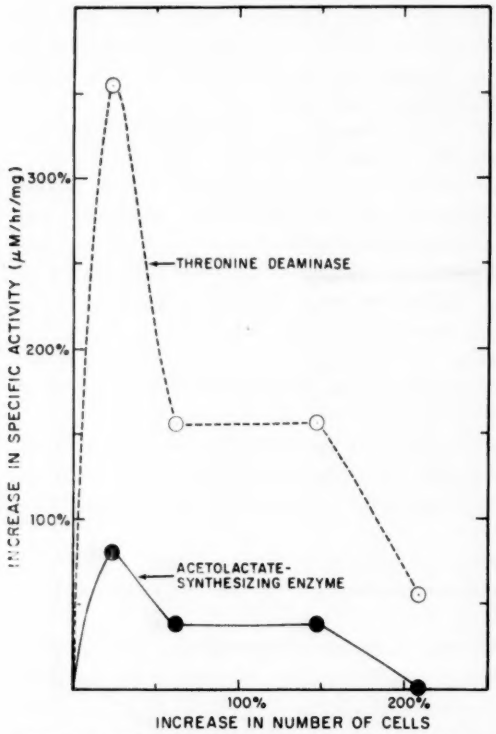

FIG. 3.—Patterns of threonine deaminase and α-acetolactate-synthesizing activities of *Salmonella* when grown in minimal medium supplemented with valine and isoleucine. The experimental procedure was the same as that described for Figure 2 with the exception that the harvested cells were suspended in minimal medium containing 2 mM each of *L*-valine and *L*-isoleucine. See *Methods* for references and details concerning the assays. The specific activities for the initial values (0%) are: 0.32 and 1.48 for the α-acetolactate-forming enzyme and the threonine deaminase, respectively.

If the repression mechanism is insensitive to total repression by the concentration of amino acids within the cell, then a pertinent question arises. What factor(s) is then responsible for the effective repression noted when *Salmonella* is grown in nutrient broth? This repression may require factor(s) other than the amino acids tested or factor(s) in addition to the amino acids. These points are currently being investigated.

In studies employing auxotrophic strains[3] the repression exerted by isoleucine, valine, and leucine is a coordinated repression for the threonine deaminase and the four enzymes of the isoleucine-valine pathway. In the present study, the repression data involve only the reductoisomerase and the dehydrase (Table 2). A second coordination has been observed between the threonine deaminase and the α-acetolactate-forming enzyme (Fig. 3). The transaminase is not involved in either of the relationships. The enzymatic data on the reductoisomerase, dehydrase, and transaminase of the *ilva* strains of *Salmonella*[16, 17] have yielded similar conclusions since a relationship between the reductoisomerase and the dehydrase, that does not include the transaminase, has been observed.

Thus, the results obtained with wild-type *Salmonella* do not include the "multi-

valent" effect of valine, isoleucine, and leucine as the major factor responsible for the repression of the pathway. The data, furthermore, serve as evidence that the repression mechanism for this interrelated metabolic pathway may well be both complex and novel. It is anticipated that future studies will aid not only in the elucidation of the mechanism but will result also in a more complete understanding of the various repression phenomena that may occur in bacteria.

Summary.—No feedback-control mechanism is noted for the isoleucine-valine pathway of *Salmonella typhimurium*. However, repression of the reductoisomerase and the α,β-dihydroxyacid dehydrase has been observed. The effect that valine, isoleucine, and leucine, alone or in combination, have on the repression of the pathway has been studied under various experimental conditions. The present results show that the "multi-valent" coordinated repression described by others for the pathway in mutant strains is not observed in wild-type, and it is proposed that the true repression mechanism is a phenomenon more complex than the described "multi-valent" repression.

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*NORMAL COUNTERPARTS TO BENCE-JONES PROTEINS: FREE L POLYPEPTIDE CHAINS OF HUMAN γ-GLOBULIN**

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Bence-Jones proteins are commonly found in patients with multiple myeloma and have been defined classically[1] as abnormal urinary proteins that have characteristic thermosolubility properties. Structural studies[2] have shown that Bence-Jones proteins are similar or identical to the light (*L*) polypeptide chains isolated from the myeloma globulin of the same patient. Taken in conjunction with the results of studies of their biosynthesis,[3,4] this finding suggests that Bence-Jones proteins are free *L* chains that have not been incorporated into the myeloma proteins. Because of their structural similarities to chains of myeloma proteins, it was predicted[5] that chains of normal γ-globulins would also have the properties of Bence-Jones proteins. Subsequently, *L* chains of 7*S* γ-globulin from normal individuals were found to resemble Bence-Jones proteins in both physicochemical[2] and antigenic[6] characteristics. Recently, γ-globulins of low molecular weight (γ_L-globulins) have been isolated from the plasma[7] and urine[8–11] of normal human subjects. These proteins were shown[12] to have close antigenic relationships to the *S* fragment, a split product of human 7*S* γ-globulin that is known to contain *L* chains.[6] It has also been found[12,13] that the γ_L-globulins contain antigenic counterparts to the two main antigenic types[14, 15] of Bence-Jones proteins.

All of the above findings suggested that the γ_L-globulins are similar to the *L* chains obtained from dissociated normal 7*S* γ-globulins and prompted the present comparative study. The results indicate that normal γ_L components and *L* chains are alike in antigenic structure, thermosolubility, conformational stability, and molecular weight. They appear to be normal counterparts to Bence-Jones proteins.

Materials and Methods.—Isolation of γ_L-globulins—Plasma γ_L-globulin: 2140 ml of pooled normal plasma was subjected to two successive ultrafiltrations, as described previously.[7] The concentrated ultrafiltrate, containing the γ_L-globulin, had a volume of 3.6 ml and a protein content of 73 mg as determined by a modified Folin procedure[16] with human albumin as standard. It was used for some of the immunochemical experiments. γ_L-globulin was purified further from a 2.2 ml aliquot of the concentrated ultrafiltrate by zone electrophoresis at pH 8.6 on a Pevikon block.[17] As a reference, normal plasma was separated on an adjacent part of the same block. The separated ultrafiltrate contained a post γ-globulin fraction and proteins corresponding to all of the main electrophoretic fractions of plasma. Material corresponding to the slow moving part of the reference plasma γ-globulin zone was concentrated to 1.2 ml by ultrafiltration, using Visking $^{2\,3}\!/_{3\,2}$ in. tubing.[18] The concentrated fraction contained 1.7 mg protein[16] representing 6–7% of the total protein recovered from the block. It contained γ_L-globulin, but was free from 7*S* γ-globulin as shown by Ouchterlony plate analyses, using antisera to γ-globulin and human serum. The preparation also contained a protein that was immunologically unrelated to γ-globulin.

Urinary γ_L-globulin: The urinary γ_L-globulin fraction used in the immunochemical experiments was obtained by submitting pooled urine from normal male subjects to two successive ultrafiltrations.[7, 11]

The γ_L-globulin preparations used in the other experiments were purified as follows: 10.0 l. of pooled normal urine was concentrated by ultrafiltration, using Visking $^{2\,3}\!/_{3\,2}$ in. tubing of comparatively low permeability.[18] 6.9 ml of the urine concentrate, corresponding to 8.2 l. of urine,

was subjected to zone electrophoresis at pH 8.6 on a Pevikon block.[17] The pattern obtained after electrophoresis was similar to that previously described.[19] Material corresponding to the γ-globulin zone was concentrated to 1.14 ml by ultrafiltration, using Visking $8/32$ in. tubing of relatively high permeability.[18] The amounts of protein in the concentrate (fraction *A*) and in the ultrafiltrate (fraction B) were determined[16] using Cohn fraction II γ-globulin as standard. The amount of protein in fraction *A* was 50 mg and in fraction *B* was 3.5 mg.

Fraction *A* contained 7*S* γ-globulin, γ_L-globulin, and glycoprotein (polysaccharide) material with a high content of fucose.[11, 18] Fraction *B* contained γ_L-globulin and relatively more of the glycoprotein (polysaccharide) material.[11, 18] A portion of fraction A containing 41 mg of protein was submitted to gel filtration on a column of Sephadex *G*-100 (2.2 cm by 46 cm)[20] in 0.05 *M* phosphate buffer, pH 7.2, + 0.2 *M* NaCl. The elution rate was 6–7 ml per hr. The elution diagram showed two well separated peaks, containing similar amounts of protein. Ouchterlony plate analyses, using anti-γ-globulin and antiserum to human serum, showed that the first peak contained 7*S* γ-globulin and the second peak γ_L-globulin and traces of 7*S* γ-globulin. The material in the second peak was concentrated and rerun on the column. The γ_L-globulin obtained was free from 7*S* γ-globulin. It contained 18 mg of protein (calculated as γ-globulin)[16] and the fucose[21] amounted to 3.5% of the protein content.

This γ_L-globulin preparation was purified further by ammonium sulfate fractionation at pH 7.5. Preliminary experiments showed that most of the γ_L preparation precipitated at saturations of ammonium sulfate between 60 and 80%. An aliquot of the γ_L-globulin preparation, containing 10 mg of protein, was saturated with ammonium sulfate to 80% at pH 7.5. The precipitate was washed once with 80% ammonium sulfate, pH 7.5, and dissolved in 3.0 ml of 0.05 *M* acetate buffer, pH 5.0, + 0.15 *M* NaCl. Some of the precipitated protein remained undissolved and was removed by centrifugation. The supernatant contained 5.4 mg of protein and its fucose content was about 1% of the protein content. Part of the preparation was dialyzed against repeated changes of the acetate buffer and was used for the nephelometric studies reported below. Some of the material was dialyzed against repeated changes of distilled water, lyophilized, and subjected to amino acid analysis.

Fraction *B*(the ultrafiltrate described above) was also purified by the same method of ammonium sulfate precipitation. The partially purified γ_L-globulin was subjected to the starch gel electrophoretic, spectrofluorometric, and molecular weight analyses described below.

In the present discussion γ_L-globulins of the plasma are denoted $\gamma_L(P)$; those of the urine are called $\gamma_L(U)$.

Isolation of L chains: Human 7*S* γ-globulin was obtained as Cohn fraction II (lot *C*-679, Lederle Laboratories, Pearl River, New York). It was purified further by zone electrophoresis on starch blocks at pH 8.6.[22] *L* polypeptide chains were obtained by reduction of the γ-globulin with 0.1 *M* β-mercaptoethanol in 8 *M* urea, followed by alkylation with iodoacetamide as previously described.[2, 5] The *L* chain fraction was isolated by chromatography on carboxymethylcellulose in 6 *M* urea.[2, 6] The fully reduced *L* chains were not soluble in aqueous buffers. To obtain *L* chains in soluble form for the immunologic and physiochemical studies, the γ-globulin was reduced in the absence of urea.[5] *L* chains were then separated from *H* chains and unreduced γ-globulin by gel filtration in Sephadex *G*-100 in 0.5 *N* propionic acid,[6] following the general procedure of Fleischman *et al.*[23]

Preparation of S and F Fragments of γ-globulin: The two immunologically distinct sets of fragments produced by proteolysis of γ-globulin with papain[24] were isolated as described in a previous publication.[25] Following the conventions adopted in that work, the fragments known to contain the antibody combining site and having a slow immunoelectrophoretic mobility are labeled *S*. The fast moving components are called *F*.

Immunologic techniques: The antisera were obtained by immunizing rabbits with the antigen in complete Freund's adjuvant. These antisera were employed in previous studies,[6] and the conventions used here are the same. For example, antiserum 509 which contained both anti-*F* and anti-*S* specificities is denoted 509 (*F*, *S*). When completely absorbed with *F* fragment, leaving only anti-*S* antibodies, it is called 509 (*S*). After complete absorption with *S* fragments it is called 509 (*F*).

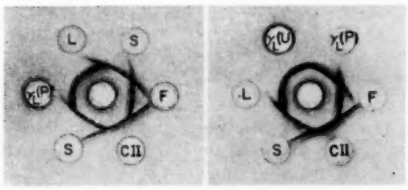
One antiserum was obtained by immunizing a rabbit according to the same procedure with *L*

chains isolated by gel filtration from partially reduced γ-globulin. It showed specificity for *L* chains and *S* fragments, but did not react with *H* chains or *F* fragments.

Immunoelectrophoresis was done by the Scheidegger micromethod;[26] the details of the procedure have been described.[11, 25] Ouchterlony plate analyses were also performed according to a previously described micromethod.[11] Photographs were taken both before and after staining the gels. The photographs reproduced here are of the stained preparations.

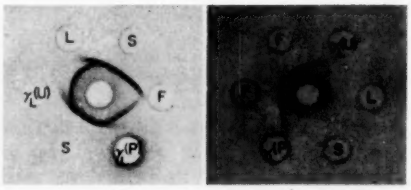
Other physical and chemical methods: Starch gel electrophoresis, spectrofluorometry, nephelometry, amino acid analysis, and ultracentrifugation were performed exactly as described in a previous publication.[2]

Results.—In Figures 1*a* and 1*b* are shown Ouchterlony plate analyses of the antigenic relations among γ_L components, *L* chains, *S* and *F* fragments, and whole γ-globulin. When tested with antisera directed against whole γ-globulin (*S,F* antisera), $\gamma_L(P)$, $\gamma_L(U)$, and *L* chains showed precipitin lines which fused completely. The distance of the lines from the antigen and antibody reservoirs was the same when equivalent amounts of antigen and antibody were used. Consonant with the findings in previous studies,[6] *L* chains, $\gamma_L(P)$, and $\gamma_L(U)$ cross-reacted with S fragments but were antigenically deficient to *S* fragments (Figs. 1*a* and 1*b*). The precipitin lines of $\gamma_L(P)$, $\gamma_L(U)$, and *L* chains crossed the lines of the *F* fragments and seemed to share no antigenic determinants with *F* fragments.



a b

FIG. 1.—Immunologic comparison of γ_L globulins, L chains, S and F fragments, and normal 7S human γ-globulin. (*a*) Ouchterlony plate analyses using γ_L (P). (*b*) Ouchterlony plate analyses using γ_L (P). and γ_L (U). Central well contained antiserum 509(F,S).



a b

FIG. 2.—Ouchterlony plate analyses using specific anti-S sera and anti-F sera. (*a*) Central well contained antiserum 506(S). (*b*) Central well contained antiserum 509(F).

Symbols over peripheral wells: γ_L (P), γ_L globulin from plasma; γ_L (U), γ_L globulin from urine; L, light polypeptide chains; S, S fragment; F, F fragment; CII, Cohn fraction II human γ-globulin.

These relations were confirmed using antisera containing anti-*S* antibodies only (Fig. 2*a*) and anti-*F* antibodies only (Fig. 2*b*). As seen in Figure 2*b*, *F* antisera failed to react with $\gamma_L(P)$, $\gamma_L(U)$, *L* chains, and *S* fragments. When an antiserum directed specifically against *L* chains was used, there was complete fusion of the precipitin lines of $\gamma_L(P)$, $\gamma_L(U)$, *L* chains, and *S* fragments, and no reaction with *F* fragments (Fig. 3).

An immunoelectrophoretic comparison of the low molecular weight γ-globulins, the *L* chains, the S and *F* fragments, and whole γ-globulin is presented in Figure 4. The median mobility of $\gamma_L(U)$ and *L* chains was similar to that of whole γ-globulin. There was a slight difference in both mobility and extent of the arcs of the γ_L component and *L* chains, the median mobility of $\gamma_L(U)$ being somewhat higher than that of the *L* chain preparation. This may have resulted from the

fact that the L chains were unavoidably exposed to reduction and alkylation. Moreover, the source of L chains was a pool of 7S γ-globulin; it is possible that $\gamma_L(U)$ contains contributions of L chains from cells making 19S- and γ_{1A}-globulins. The widely differing mobilities of S and F fragments are seen in Figure 4. Despite their close antigenic relations, the mobility of S fragments was less than that of L chains.

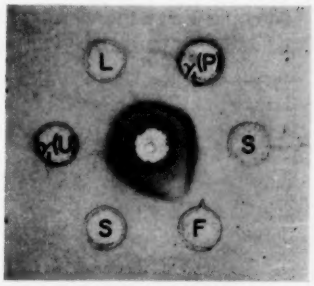


FIG. 3—Ouchterlony plate analysis using an antiserum directed against L polypeptide chains. Symbols over peripheral wells as in Figure 1 and text.

Starch gel electrophoresis in 8 M urea of reduced alkylated 7S γ-globulin separates the L chains from the H chains and undissociated material.[2, 5] Partially purified $\gamma_L(U)$, after reduction and alkylation, showed a diffuse band distribution similar to that of the L chains (Fig. 5). A partially purified L chain preparation examined on the same gel was applied at the same concentration as the reduced alkylated γ-globulin. This accounts for the intense staining and apparently broader distribution of this material.

The small amounts of γ_L components available prevented a direct assessment of their sedimentation velocity by schlieren methods. A molecular weight analysis of $\gamma_L(U)$, using the method of equilibrium sedimentation described by Yphantis,[27] yielded a value of 25,000 in a solvent of phosphate buffer pH 7.5, $\Gamma/2$ 0.05, made 0.15 N in KCl. This figure agrees with the measured values for L chains in 6 M urea and apparently is the approximate molecular weight of the monomer. The $\gamma_L(U)$ preparation also contained 20–25% of materials with molecular weights of 4,000–6,000.

Limitations on the amount of material presently available also precluded a determination of an accurate absolute amino acid composition of the γ_L components. The relative composition[5] per mole of alanine in one $\gamma_L(U)$ preparation and L chains is given in Table 1. Although both preparations showed the same amounts of lysine, proline, valine, isoleucine, leucine, and tyrosine, they contained different amounts of the other amino acids.

The property that serves to distinguish Bence-Jones proteins from all other known proteins is their precipitation between 48°–60°C, followed by redispersion at higher temperatures. In many instances this behavior is completely or partially reversible. In Figure 6 is given a qualitative nephelometric analysis of a $\gamma_L(U)$ preparation. The aggregative behavior and thermosolubility properties are those of Bence-Jones proteins and L chains.[2]

A more precise characterization of the conformational changes that occur upon heating a protein may be obtained by spectrofluorometry.[28] In Figure 7, thermally induced molecular transitions characteristic of Bence-Jones proteins[2] are shown by L chains and $\gamma_L(U)$ globulin. The transition temperatures differed by 1°C. Although this difference is at the limits of precision of the method, it is probably real, reflecting the difference in origin, purity, and chemical treatment of the two preparations. It is notable that the $\gamma_L(U)$ transition was partially reversible as are those of the majority of Bence-Jones proteins.

Discussion.—The foregoing results leave little doubt that γ_L components and

L chains are similar if not identical. Their antigenic identity appears to be established by the Ouchterlony plate analyses; their close antigenic relation to the two main antigenic types of Bence-Jones proteins was ascertained in previous studies.[12,6] The slight differences observed in the immunoelectrophoretic patterns and starch gel patterns remain to be explained. As mentioned above, they probably arise from the different origins of the γ_L and L chains, as well as from an unavoidable fractionation that occurs in isolating these materials. Because of the inherent heterogeneity of normal L chains[5] and γ_L components, a complete proof of their identity remains presently beyond reach.

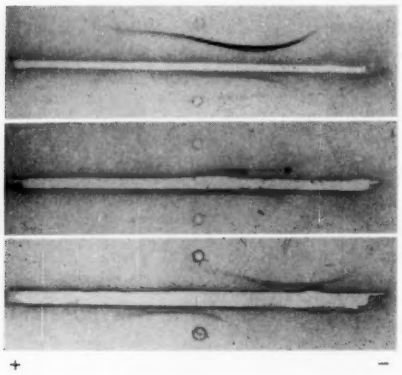


FIG. 4.—Immunoelectrophoretic comparison of γ-globulin, γ_L components, L chains, and fragments produced by hydrolysis with papain. γ, 7S γ-globulin; other symbols as in Figure 1. The lines were developed using rabbit (F,S) antiserum.

The physicochemical studies confirm that $\gamma_L(U)$ components have the thermosolubility properties of Bence-Jones proteins. Although the general spectrofluorometric behavior of $\gamma_L(U)$ globulins was that of L chains, there were significant differences in the shapes of the curves. These differences may have reflected differences in molecular aggregation and purity of these preparations. The predominant component in the $\gamma_L(U)$ preparation examined had a molecular weight of 25,000. Previous studies[2] have shown that L chains isolated from normal 7S γ-globulin are present in aqueous solutions mostly as dimers with molecular weights of 40,000 but also as polymers of higher molecular weight.

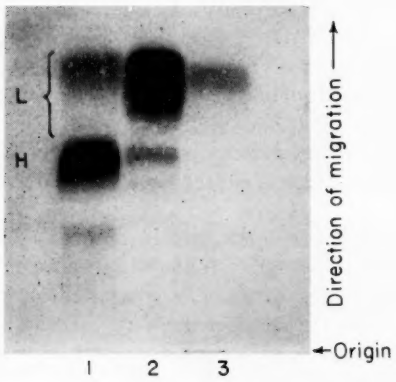


FIG. 5.—Starch gel electrophoretic comparison of γ_L (U) globulin, L chains, and dissociated 7S human γ-globulin. 1, Dissociated γ-globulin; 2, Partially purified L chains; 3, γ_L(U) globulin. L, light chains; H, heavy chains.

Contaminating low molecular weight materials were seen in the ultracentrifugal analysis of $\gamma_L(U)$. The materials were probably peptides, since the amino acid analyses showed distinct differences between the $\gamma_L(U)$ preparation and L chains. It is notable, however, that the relative amino acid content of the two preparations was the same for many of the stable amino acids. A number of variables could have influenced the relative composition of $\gamma_L(U)$ and L chain preparations. They include selective filtration by the kidney, contamination by glycopeptides, differences in biosynthetic rates, and differential susceptibility

TABLE 1

RELATIVE AMINO ACID CONTENTS OF A $\gamma_L(U)$ GLOBULIN PREPARATION AND L POLYPEPTIDE CHAINS OF HUMAN 7S γ-GLOBULIN*

Amino acid	$\gamma_L(U)$ globulin†	L chains‡
Lysine	0.790	0.806
Histidine	0.230	0.174
Arginine	0.580	0.444
Aspartic acid	1.25	1.06
Threonine	0.890	1.12
Serine	1.49	1.58
Glutamic Acid	1.73	1.55
Proline	0.890	0.890
Glycine	1.09	1.02
Alanine	1.00	1.00
Valine	1.05	1.07
Methionine	0.131	0.052
Isoleucine	0.400	0.405
Leucine	1.14	1.10
Tyrosine	0.585	0.690
Phenylalanine	0.550	0.482

* All values expressed as moles per mole of alanine.
† Isolated by gel filtration and ammonium sulfate precipitation.
‡ Isolated from partially reduced alkylated 7S human γ-globulin by gel filtration.

to acid hydrolysis. Further studies will be required to assess the relative contribution of each factor.

A fundamental problem raised by the findings is whether free L chains in the plasma and urine are degradation products of normal γ-globulin or are as in the case of Bence-Jones proteins,[3, 4] precursors or by-products of γ-globulin synthesis. On evidence obtained using radioactive tracers, both Webb *et al.*[29] and Franklin[9] concluded that the low molecular weight urinary proteins are degradation products of normal γ-globulin. On the other hand, Stevenson[13] found the kinetics of elimination of tracer labeled products to be incompatible with degradation. The components described by Stevenson apparently are the same as the $\gamma_L(U)$ components described by one of us,[7] and it appears most likely that free L chains in normal plasma and urine arise from "de novo" synthesis.

If confirmed, this would appear to be the first instance described of asynchronous or unbalanced chain production in normal multichain proteins. Similar asynchrony has been described in the synthesis of abnormal hemoglobins, e.g. hemoglobin H.[30–32] In this case it appears to result from repression of genes controlling α-chains. In the case of the normal γ-globulins, the excess of L chains may arise merely as an inevitable consequence of the possibility that L chains are under control of a much larger set of structural genes than are H chains.[33] It may also be that H chain production depends upon the level of L chain synthesis. In this connection, it would be pertinent to establish whether the low molecular weight γ-globulin found in the plasma of newborn piglets preceding the production of 7S γ-globulin[34] represents free L chains.

The physiologic significance of excessive L chain production is not apparent. It is worthwhile to focus attention on several connected observations that may bear upon the problem. Guinea pig antibodies of different specificity appear to contain L chains of different structure.[35] Moreover, the L chains are contained in the S fragment[6] which is known to possess the antibody combining site. The possibilities that specific L chains may have antibody activity[36] and may be involved in the regulation of antibody synthesis should be explored. L chains

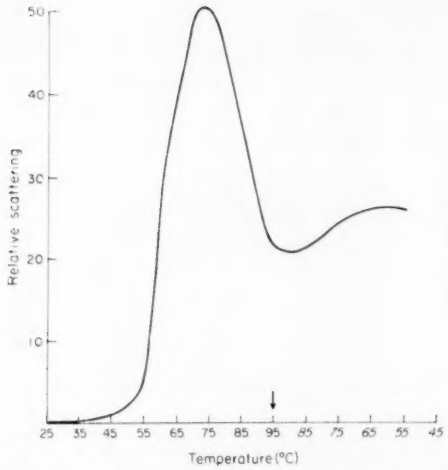


FIG. 6.—Thermosolubility properties of γ_L(U) globulin as detected by nephelometry.

Ordinate: relative scattering of monochromatic light (546.1 mμ) measured at 90° to incident beam.

Solvent: sodium acetate buffer 0.05 *M*, pH 5.0, + 0.15 *M* NaCl.

Protein concentration: 0.5 mg/ml.

↓: cessation of heating at 1°/min. Cooling at undetermined rate.

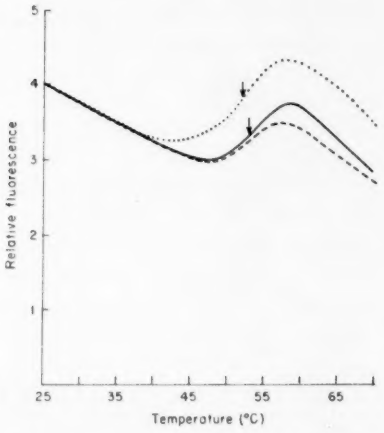


FIG. 7.—Thermally induced molecular transitions of γ_L (U) globulin and L chains detected by spectro-fluorometry. ——γ_L (U) globulin. - - - γ_L (U) reheated once. . . . L chains.

↓: Transition temperature. Solvent: sodium phosphate buffer, pH 7.05, ionic strength 0.2. Protein concentration: 0.12 mg/ml.

appear to be the common structural element[6, 33] possessed by all γ-globulins of different classes (γ, γ_{1A}, γ_{1M}), further supporting the notion that they play a central role in the acquisition of immunologic specificity.

Summary.—Low molecular weight γ_L-globulins isolated from normal human urine and plasma were found to resemble the *L* polypeptide chains of normal human 7*S* γ-globulin in their physicochemical and antigenic behavior. Both *L* chains and γ_L components have the thermosolubility properties and spectrofluorometric behavior of Bence-Jones proteins. It is suggested that γ_L globulins are free *L* chains and that they are normal counterparts to Bence-Jones proteins.

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*BACTERIAL PHOTOPHOSPHORYLATION: REGULATION BY REDOX BALANCE**

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Pigmented particles derived from photosynthetic bacteria catalyze light-dependent synthesis of ATP[1] from ADP and P_i and also manifest a number of light-stimulated oxidation-reduction reactions. The *in vitro* photophosphorylation process first demonstrated by Frenkel[2] occurs readily in the absence of external electron acceptors and, in further contrast with oxidative phosphorylation, does not require addition of electron donors in significant quantity. The over-all reaction is generally interpreted to represent phosphorylation coupled with a closed circuit transfer of photochemically-generated electrons, or hydrogen atoms, through an electron transfer chain to an oxidant (presumably oxidized bacteriochlorophyll),

which is also created by the photoexcitation. Antimycin A is a potent inhibitor of the light-dependent ATP synthesis, which is commonly referred to as "cyclic photophosphorylation."

Existence of a second type of bacterial photophosphorylation, dependent on light-stimulated *net* electron transfer between added electron donor and acceptor systems, has recently been claimed by Nozaki, Tagawa, and Arnon.[3] According to their interpretation, pigmented particles catalyze an antimycin-resistant photophosphorylation coupled with "noncyclic" electron flow between ascorbate + DCPIP (electron donor) and NAD (acceptor); NAD is presumably reduced by electrons ejected by photoexcitation of bacteriochlorophyll and the oxidized pigment is restored to its original state by electrons from ascorbate + DCPIP. We have recently suggested[4] that a more plausible explanation of the light-stimulation of certain oxidation-reduction reactions in these organisms (or particles obtained therefrom) is the promotion of "dark" reactions by energy-rich intermediates produced through the interaction of light with the photochemical apparatus, i.e., energy-dependent electron transfer of the kind observed by Chance and Hollunger[5] in mammalian mitochondria. In this communication, we summarize evidence supporting the view that the apparent "noncyclic" phosphorylation reported by Nozaki *et al.*[3] is in reality cyclic photophosphorylation, which is antimycin-resistant due to the ability of DCPIP to effect a "by-pass" in light-induced cyclic electron transfer. In addition, the results confirm and extend the observation, due mainly to Kamen and colleagues,[6, 7] that optimal activity of the cyclic photophosphorylation system in isolated pigmented particles is dependent on maintenance of a suitable redox potential.

Materials and Methods.—Growth of bacteria: *Rhodospirillum rubrum* (strain Sl) was grown photosynthetically in a synthetic medium containing succinic acid (6 gm/liter), and L-glutamic acid (1 gm/liter) as the sole nitrogen source. Other ingredients of the medium and the general procedures used in growing the cultures were as described by Ormerod *et al.*,[8] except that a gas phase was not used.

Preparation of pigmented particles: Cells were harvested after 62–65 hr of growth, washed twice with 0.005 *M* potassium phosphate buffer pH 7.6, and ground[9] with alumina (Alcoa A-301). The disrupted cells were extracted with the phosphate buffer noted, and alumina, unbroken cells, and large subcellular fragments removed by low-speed centrifugations. Pigmented particles were collected from the supernatant fluid by centrifugation at 59,000 × *g* for 30–45 min. The particles were washed twice and stored in concentrated suspension in the phosphate buffer, under an atmosphere of H_2, in an ice bath. All operations during preparation of the cell-free extracts and particle suspensions were carried out at 0°–4°C.

Incubation conditions and analytical methods: The experimental suspensions were placed in Warburg vessels which were shaken in a bath equipped with a glass bottom. Illumination was provided, from below, by 50-watt reflector flood lamps. For most of the experiments, as noted, a sheet of red cellophane (total cutoff of light <555 mμ) was inserted between the lamps and the glass window. The light intensity, without the red filer, was 1500 foot-candles as measured with a General Electric light meter. Dark controls were simultaneously run in a shielded portion of the same bath.

Light-induced phosphorylation (LIP) of adenosine diphosphate was determined by measuring the disappearance of P_i in the presence of Mg^{++}, hexokinase, and glucose; ADP and hexokinase were added to the other components at zero time. To terminate the reactions, trichloroacetic acid was added to give a final concentration of 3.5%. P_i in the deproteinized fluids was determined by the method of Taussky and Shorr[10] and the results expressed as Light-Dark differences. Control experiments showed that addition of the various supplements used (ascorbate, DCPIP, etc.) did not induce phosphorylation in darkness.

Bacteriochlorophyll, in the pigmented particles, was determined by the method of Cohen-Bazire *et al.*[11]

Results.—Effects of reducing and oxidizing agents on LIP: Earlier studies[2, 12] with washed pigmented particles from *R. rubrum* disclosed that addition of very small quantities of certain organic substrates (e.g., succinate, lactate, α-ketoglutarate, $NADH_2$) caused activation of LIP under the usual conditions of anaerobic assay. This substrate effect has been frequently attributed to removal, by dehydrogenase action, of inhibitory traces of contaminating oxygen. On the other hand, the activation of LIP by reducing agents has been ascribed[6, 7, 13] to establishment of an optimal redox potential of one or more participating electron carriers. Our experiments support the conclusion that the effects of oxidizable organic substrates are primarily concerned with adjustment of the redox potential to a region required for maximal LIP. The redox potential can apparently also be shifted to this region by molecular hydrogen, as indicated by the data of Table 1.

TABLE 1

EFFECTS OF AIR AND HYDROGEN ON LIGHT-INDUCED PHOSPHORYLATION

Gas phase	P_i utilized (μmoles)
Argon	1.7
Helium	1.9
Hydrogen	3.9
Air	0

The reaction mixtures contained in a final volume of 3.0 ml: Tris buffer, pH 8.0, 100 μmoles; $MgSO_4$, 5 μmoles; K_2HPO_4, 10.2 μmoles; ADP, 0.5 μmole; glucose, 30 μmoles; hexokinase, 1 mg; particles, equivalent to 0.12 mg bacteriochlorophyll. Temperature, 30°C; incubation time, 15 min.

Under an atmosphere of H_2, LIP is significantly stimulated in comparison with the activity observed under argon or helium. Furthermore, the stimulatory effect of H_2 is catalytic in the sense that it is exerted in the absence of manometrically detectable utilization of H_2. The particulate preparations used in the present studies contain an active hydrogenase which is capable of oxidizing H_2 with a variety of electron acceptors (e.g., PMS, DCPIP, *p*-benzoquinone, ferricyanide).[4, 14] In view of the evidence[7] that *c*-type cytochromes act as electron carriers in LIP, it is particularly noteworthy that *R. rubrum* particles can also reduce mammalian cytochrome *c* through hydrogenase action.[15]

It appears that the degree of inhibition of LIP by oxygen is dependent on the vigor of aeration and can be significantly diminished by the presence of metabolizable substrates[16, 17] or reducing agents.[7] In our experience, with well-shaken reaction mixtures, virtually complete inhibition is observed upon aerobic incubation when reducing substances are not added (Table 1).

Displacement of the redox potential; "over-reduction" and "over-oxidation": If a critical redox range is required for optimal LIP, it is evident that appreciable displacement of the potential should eventually lead to inhibition. LIP by *R. rubrum* particles is, in fact, inhibited when certain "exogenous" electron donors (e.g., succinate, $NADH_2$, ascorbate) are present in excess.[7, 17] Inhibition by "over-reduction" is particularly marked if the experimental system also contains a redox dye (PMS or DCPIP) which can readily interact with components of the photochemical electron transfer chain. Thus, partially reduced PMS is an activator of LIP, but the dye becomes a strong inhibitor when it is maintained in the reduced state by an appropriate reducing agent.[12] These and similar results ob-

served with *Chromatium* particles[6] indicate that efficient LIP is dependent on maintenance of redox components of the phosphorylating system at particular oxidation-reduction potentials.

The experiment summarized in Table 2 illustrates the effect of over-reduction

TABLE 2

INHIBITION OF LIGHT-INDUCED PHOSPHORYLATION BY OVER-REDUCTION WITH H_2

Additions	Gas phase	P_i utilized (μmoles)
None	A	5.0
Ascorbate (0.2 μmole)	A	5.7
Ascorbate + DCPIP (0.2 μmole)	A	7.9
None	H_2	7.6
Ascorbate (0.2 μmole)	H_2	7.3
Ascorbate + DCPIP (0.2 μmole)	H_2	3.3

When both ascorbate and DCPIP were used, they were mixed in a side-arm of the vessel while the latter was being gassed. The additions were introduced at zero time. Other experimental conditions as in Table 1, except that the particles contained 0.11 mg bacteriochlorophyll and the incubation time was 30 min.

on LIP. In the control series, under argon, 6.6×10^{-5} *M* ascorbate accelerated LIP slightly while a pronounced stimulation was caused by addition of DCPIP, exactly titrated with the same concentration of ascorbate. LIP by *Chromatium* particles is similarly activated by ascorbate + DCPIP mixtures.[6]

With H_2 as the gas phase, LIP proceeded at the "activated" rate in the absence of other adjuvants. Addition of ascorbate alone had little effect, but the ascorbate + DCPIP mixture was inhibitory. These results can be explained by assuming that, in the presence of H_2, the hydrogenase tends to keep the dye in the reduced state and, thereby, shifts the over-all redox potential of the particles to an unfavorable (reduced) position. In the absence of H_2, the dye would presumably remain in a more oxidized state, particularly because the particles catalyze a rapid anaerobic photooxidation of reduced DCPIP with endogenous electron acceptors.[18] It is evident that in systems containing DCPIP exactly titrated with ascorbate, the "extra" reducing effect of H_2 creates a redox situation comparable to that existing when DCPIP + excess ascorbate are present under an inert gas. Accordingly, we have found that if the ascorbate/DCPIP ratio exceeds 2, LIP under argon is inhibited.

The extent of inhibition of LIP by over-reduction with H_2 is, within limits, dependent on the hydrogenase content of the system or on the activity of the enzyme under the particular conditions used (see discussion of Table 6). This can be readily demonstrated by increasing the enzyme level with an exogenous source of hydrogenase as shown in Table 3.

TABLE 3

OVER-REDUCTION OF THE PHOTOPHOSPHORYLATION SYSTEM BY AN EXOGENOUS HYDROGENASE

Additions	Gas phase	P_i utilized (μmoles)
None	H_2	5.2
DCPIP (0.2 μmole)	H_2	5.5
Escherichia coli particles (protein $\equiv$ 300 μg)	H_2	3.2
E. coli particles + DCPIP	H_2	0.5
E. coli particles + DCPIP + fumarate (20 μmoles)	H_2	1.8
None	A	4.1
E. coli particles	A	4.2

The dye, when used, was reduced in darkness with H_2 by the hydrogenase(s) prior to initiation of the LIP reaction. Hydrogenase-containing particles from *E. coli* were prepared as described in ref. 19. Other conditions as in Table 1, except that the *R. rubrum* particles contained 0.13 mg bacteriochlorophyll and the reaction time was 20 min in red light.

Ascorbate was not added in the trials summarized in Table 3, and the results therefore reflect the effects of reducing activity primarily due to hydrogenase. Addition of only DCPIP to the *R. rubrum* particles had a negligible effect on LIP in this particular experiment, but presence of the dye frequently enhances the activity. In the absence of the dye, the particulate *Escherichia coli* hydrogenase caused an appreciable inhibition of LIP; separate tests showed complete inhibition when the quantity of *E. coli* particles was increased 2.5 fold. It can be seen that LIP was not suppressed by the *E. coli* particles when an argon atmosphere was used. Accordingly, it seems probable that the remarkable inhibition observed under H_2 is caused by over-reduction of electron carriers of the *R. rubrum* LIP system by the *E. coli* hydrogenase. When both DCPIP and the exogenous hydrogenase were present. LIP was almost completely suppressed and inclusion of fumarate in the reaction mixture led to a definite relief of the inhibition; the mechanism of this type of reversal is discussed below.

It might be expected[6] that extensive displacement of the redox potential by "over-oxidation" would also cause inactivation of the LIP system, and the inhibitory effects of oxidized dyes,[12] ferricyanide,[20] and oxygen can be rationalized on this basis.

Antimycin-sensitivity of LIP: The LIP activity of *R. rubrum* particles, in the absence of dyes or other adjuvants, is very sensitive to inhibition by antimycin A.[12] Supplementation with partially reduced PMS, however, accelerates LIP activity and also confers antimycin-resistance on the system. These observations suggest[12] that when the oxidation-reduction potential of the system is properly adjusted, PMS can act as a redox carrier which by-passes a rate-limiting antimycin-sensitive step. The results of Table 4 show that under an atmosphere of H_2, the dye DCPIP also catalyzes antimycin-resistant LIP.

TABLE 4

ANTIMYCIN-RESISTANCE OF LIGHT-INDUCED PHOSPHORYLATION IN THE PRESENCE OF DCPIP

Additions	P_i utilized (μmoles)
None	9.9
Antimycin A (10 μg)	0
DCPIP (0.2 μmole)	10.6
DCPIP + antimycin A	7.8

Gas phase, H_2. Other conditions as in Table 3.

The foregoing results, and those of similar experiments with particles from malate-grown cells, indicate that PMS and DCPIP interact with the LIP system in essentially the same way. Both dyes are readily reduced by the particulate *R. rubrum* hydrogenase,[4] and the similarity of their behavior is further illustrated by the experimental results of Table 5.

TABLE 5

OVER-REDUCTION OF THE PHOTOPHOSPHORYLATION SYSTEM IN THE PRESENCE OF PMS

Additions	P_i utilized (μmoles)
None	6.0
Antimycin A (10 μg)	0.7
PMS (1 μmole)	5.1
PMS + antimycin A	3.4
PMS + ascorbate (1 μmole)	0.2

Experimental conditions as in Table 4.

With only PMS added, LIP was partially suppressed, presumably due to slightly adverse displacement of the redox potential resulting from hydrogenase activity; the phosphorylation was, as expected, largely antimycin-resistant. The simultaneous presence of H_2, PMS, and a low concentration of ascorbate led to essentially complete inhibition of LIP. These results indicate that the *R. rubrum* particles become easily over-reduced when either PMS or DCPIP (see Table 2) plus an equivalent amount of ascorbate are present together with H_2. It would appear, furthermore, that under conditions of over-reduction, the dyes cannot effectively catalyze the antimycin-resistant by-pass discussed earlier.

Adjustment of the redox potential by added reductants and oxidants: The LIP system of isolated pigmented particles evidently is weakly "poised," i.e., shows comparatively low resistance to displacement of the redox potential.[21] Shift of the potential to the reduced side, and its attendant effects on LIP, should therefore be easily reversed by addition of oxidants. Vernon and Ash[17] have accordingly shown that fumarate or NAD (plus pyruvate and lactic dehydrogenase) can prevent the over-reduction effect of excess succinate or ascorbate + DCPIP on LIP by *R. rubrum* particles. Concurrently, a *net* light-stimulated electron flow between added reductant (e.g., ascorbate + DCPIP) and oxidant (fumarate or NAD) can also be demonstrated. Similarly, if a sufficiently high concentration of particles is used, light-stimulated consumption of H_2 occurs with fumarate as the ultimate electron acceptor when DCPIP or certain other redox mediators of relatively high potential are present.[4] Nozaki *et al.*[3] have concluded that the occurrence of antimycin-resistant phosphorylation during net electron transfer between ascorbate + DCPIP and NAD constitutes evidence for the existence of a "noncyclic" photophosphorylation mechanism, i.e., a phosphorylation coupled with the light-stimulated oxidation-reduction reaction noted. The data of Table 6, however, show that antimycin-resistant phosphorylation, *dependent on the presence of both reductant and oxidant*, can occur in the absence of net electron flow.

In experiment I (at 30°C), the inhibitory over-reduction effect of H_2 + ascorbate + DCPIP was completely reversed by addition of fumarate. In fact, there was a definite stimulation of LIP as compared with the "cyclic" photophosphorylation control (i.e., the trial with no additions). LIP in the "restored" system was, furthermore, quite resistant to inhibition by antimycin A. It is of importance to note that appreciable net electron transfer did not occur in the "complete" system, i.e., 8.5 μmoles of P_i were esterified in the presence of only 0.2 μmole of reducing agent and H_2 consumption was not detectable.

Experiment II was conducted at 20°C in view of Vernon's observation[18] that this temperature is optimal for NAD-dependent photooxidation of reduced DCPIP by *R. rubrum* particles. At 20°C, the *R. rubrum* hydrogenase is practically inactive and inhibition of LIP by over-reduction was consequently not observed. Under the redox conditions obtaining with ascorbate + DCPIP present, the LIP activity was somewhat enhanced and was completely resistant to antimycin. Further addition of a small quantity of NAD caused pronounced stimulation of LIP and this system also showed a higher rate of phosphorylation in the presence of antimycin than the "cyclic" control. Basically similar results have been obtained in experiments using a trapping system for $NADH_2$ (viz., pyruvate and

dialyzed lactic dehydrogenase). As in experiment I, there was no appreciable net oxidation-reduction under the conditions employed for experiment II.

TABLE 6

ACTIVATION OF ANTIMYCIN-RESISTANT CYCLIC PHOTOPHOSPHORYLATION BY DCPIP AND "REDOX BUFFERS"

Experiment	Temperature	Additions	P_i utilized (μmoles)
I	30°C	None	7.5
		Antimycin A (10 μg)	0.7
		Ascorbate (0.2 μmole) + DCPIP (0.2 μmole)	0.7
		Ascorbate + DCPIP + fumarate (10 μmoles)	8.5
		Ascorbate + DCPIP + fumarate + antimycin A	4.4
II	20°C	None	4.4
		Antimycin A (10 μg)	0
		Ascorbate (0.2 μmole) + DCPIP (0.2 μmole)	5.3
		Ascorbate + DCPIP + antimycin A	5.0
		Ascorbate + DCPIP + NAD (1 μmole)	7.5
		Ascorbate + DCPIP + NAD + antimycin A	5.1

In experiment I, the particles contained 0.11 mg bacteriochlorophyll; in experiment II, 0.20 mg. Other conditions as in Table 4, except that the incubation time was 30 min.

The results shown in Table 6 again emphasize the ability of DCPIP to catalyze an antimycin-resistant by-pass in the LIP mechanism when the redox potential is suitably adjusted. We conclude that the apparent "requirement" for both (added) reductants and oxidants can be explained by their action in establishing a redox environment which permits efficient operation of cyclic LIP.

Discussion and Summary.—The present results, together with those of earlier investigations,[6, 7, 12, 13, 17] provide strong evidence for the conclusion that an optimal oxidation-reduction potential is required for maximal coupling of light-activated electron transport to phosphorylation processes. This prominent characteristic and the marked sensitivity of isolated particles to displacement of the redox potential imply that either the photochemical apparatus, *in vivo*,[23] can be maintained within a restricted redox range, or that the rate of photophosphorylation is normally subject to metabolic regulation by changes in the temporal concentrations of certain physiological reductants and oxidants. In this connection, it is of interest that extensive shift of the redox potential, with consequent inhibition of phosphorylation, can apparently be induced in intact cells of *R. rubrum* by addition of low concentrations of certain redox dyes; PMS, thionine and benzyl viologen cause inhibition of photometabolism and, under these conditions, the cells resort to a "dark" fermentation of endogenous reserves (with the formation of fatty acids) even though they are under continuous illumination.[27] This striking metabolic effect, which is also caused by antimycin A, indicates that photophosphorylation activity inhibits fermentation and, in this sense, the induced transition could be said to represent a "photosynthetic Pasteur effect."

Our experiments indicate that, under certain conditions, DCPIP can act as an efficient electron carrier which by-passes an antimycin-sensitive step, or region, of the cyclic photophosphorylation system of isolated particles. This activity of the dye is abolished, however, if it is maintained primarily in the reduced state by an excess of reducing agent (either ascorbate or ascorbate + H_2). "Over-reduction" (or "over-oxidation") results in inhibition of phosphorylation, presumably due to severe displacement of the redox potential of one or more components of the electron transfer chain and, in systems containing DCPIP, to a

requirement for a particular steady-state ratio of oxidized to reduced dye for effective carrier action. The dye PMS also enables particles from *R. rubrum* to catalyze an antimycin-resistant photophosphorylation and, from all indications, both PMS and DCPIP affect the phosphorylation system in the same manner.

Photophosphorylation by isolated particles is activated by suitably adjusting the redox potential with mild reducing systems; this can be readily achieved through hydrogenase action or by addition of ascorbate alone. The inhibition caused by "over-reduction" with external electron donors can be effectively reversed by supplementation with certain electron acceptors, e.g., fumarate or NAD. Restoration of photophosphorylation by this means can occur *in the absence of net electron transfer* between the exogenous donor and acceptor systems. It seems reasonable to conclude that addition of the electron acceptor has the primary effect of adjusting the redox potential to an electrochemical range which is optimal for the operation of cyclic photophosphorylation. If dyes such as PMS or DCPIP are present under favorable redox conditions, the phosphorylation becomes resistant to antimycin A, apparently because the antibiotic-sensitive step is circumvented. The postulated[3] existence of "noncyclic" photophosphorylation is ultimately based on the observation of antimycin-resistant phosphorylation in experimental systems containing DCPIP as well as added reductants and oxidants. It is evident from the results described that the criterion of antimycin-resistance does not necessarily provide an adequate basis for establishing the occurrence of a process of this kind.

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[1] Abbreviations used: LIP, light-induced phosphorylation; ADP, adenosine diphosphate; ATP, adenosine triphosphate; P_i, inorganic phosphate; NAD, nicotinamide adenine dinucleotide; $NADH_2$, reduced NAD; DCPIP, 2,6-dichlorophenolindophenol; PMS, N-methyl phenazonium methosulfate; Tris, tris(hydroxymethyl)aminomethane.

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[23] Several types of evidence[24, 25] indicate that the photochemical apparatus of *R. rubrum* is normally integrated with the cytoplasmic membrane, or membranous extensions penetrating the cytoplasm. Disengagement of the pigment system, with its associated electron transfer carriers, from the native cellular matrix might be expected[6, 26] to cause a number of changes, including disturbance of redox balance.

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*A COMPARISON OF CLOSTRIDIAL FERREDOXINS**

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Mortenson, Valentine, and Carnahan[1] have recently isolated a non-heme iron-containing protein from *Clostridium pasteurianum* that is required for the formation of acetyl phosphate and hydrogen from pyruvate by extracts of that organism. They have named this protein "ferredoxin". Valentine, Jackson, and Wolfe[2] have reported that ferredoxin is also necessary for hydrogen formation in the fermentation of xanthine by *Micrococcus lactilyticus*. Tagawa and Arnon[3] subsequently crystallized *C. pasteurianum* ferredoxin and found it to have a molecular weight of about 12,000, 10 atoms of iron per molecule, and a redox potential of -418 mV at pH 7.11. Ferredoxin was also shown to be reversibly oxidized and reduced and to be similar to the "photosynthetic pyridine nucleotide reductase" originally isolated from spinach by San Pietro and Lang.[4]

Because of our interest in the metabolism of pyruvate by *Clostridium acidi-urici* and *Clostridium cylindrosporum*,[5] we examined these purine-fermenting organisms for ferredoxin and found them to contain relatively high concentrations of this protein. In the present communication, the isolation of crystalline ferredoxin from *C. acidi-urici* is described and its properties are compared with those of crystalline ferredoxin isolated from *C. pasteurianum*.

Materials and Methods.—Organisms: *C. acidi-uridi*[6] was grown on uric acid as previously described.[7] *Clostridium butyricum* was grown on glucose as described by Wolfe and O'Kane,[8] and *C. pasteurianum* was grown on sucrose with nitrogen gas as the sole nitrogen source as described by Carnahan and Castle.[9]

Determination of ferredoxin activity: Ferredoxin was assayed by measuring its stimulation of acetyl phosphate formation from pyruvate with a ferredoxin-free *C. pasteurianum* "clastic system" based on that described by Mortenson, Valentine, and Carnahan.[1] The assay mixture (1 ml) contained 10 μmoles of sodium pyruvate, 25 μmoles of potassium phosphate buffer, pH 6.5, 0.05 μmoles of CoA, 8 mg of the lyophilized DEAE-cellulose treated bacterial extract, and ferredoxin as indicated. The assay mixture was incubated at 37° for 10 min when the reaction was stopped by the addition of 0.5 ml of neutralized hydroxylamine,[10] and after an additional 10 min period, 1.5 ml of ferric chloride reagent was added.[10] The mixture was centrifuged and the absorbancy of the supernatant solution was determined at 540 mμ. The assay was linear using up to 5 μg of the crystalline *C. pasteurianum* ferredoxin, with a proportionality constant of approximately 0.055 absorbancy units per 1 μg protein[11] per ml incubation mixture per 10 min. A unit of ferredoxin activity is defined as the amount needed to effect a change of 1.0 in absorbancy at 540 mμ when corrected for the absorption of the control tube incubated in the absence of ferredoxin, and is equivalent to the formation of approximately 5 μmoles of acid hydroxamate per 10 min.

Other methods: Total iron was determined by the *o*-phenanthroline method[12] in a final volume of 2.5 ml after hydrolysis of the sample for 10 min in 1% HCl at 80°. "Labile sulfide" was determined by the method of Fogo and Popowsky,[13] using a final assay volume of 2.5 ml instead of 250 ml and measuring the absorbancy of the methylene blue formed at 670 mμ. Control experiments with cysteine, oxidized and reduced glutathione, insulin, and bovine serum albumin were negative and indicated that the method was specific for free sulfide. For the analysis of amino acids other than tryptophan, ferredoxin was hydrolyzed for 12 hr in 6.0 *N* HCl at 120°. The amino acid composition of the hydrolysate was determined with the Spinco automatic analyzer. Tryptophan was determined by fluorescence analysis following alkaline hydrolysis.[14]

Isolation and crystallization of ferredoxin: The following procedure was used for purifying ferredoxin from *C. acidi-urici* and is typical of that used for the isolation of ferredoxin from other organisms. All steps of the purification procedure were done at 2°–3°.

Preparation of cell-free extract: 230 gm of a frozen cell paste of *C. acidi-urici* were thawed overnight and suspended in water with the aid of a magnetic stirrer to a final volume of about 750 ml. Small amounts of deoxyribonuclease and ribonuclease were added to obtain a free-flowing suspension. 80 ml aliquots of this suspension were subjected to a 10 kc Raytheon Sonic Oscillator for 10 min; cell debris was removed by a 15 min centrifugation at 13,000 × *g*. The precipitate was washed with 100 ml of water by centrifugation and discarded. The wash water was added to the initial extract.

First DEAE-cellulose column: This step was based on the procedure of Mortenson, Valentine, and Carnahan.[1] The DEAE-cellulose was equilibrated with 1.0 *M* potassium phosphate buffer, pH 6.5 and was washed with water before use. A column containing approximately 75 ml of DEAE-cellulose (2.8 × 12 cm) was packed under pressure (4.5 lbs/in²). Subsequent operations were carried out under this pressure. The sonic extract was applied to the column, which was then washed with water until the eluate was colorless (approximately 10 column volumes). The major portion of the ferredoxin was adsorbed as a black band near the top of the column. The column was then washed with 10 volumes of 0.05 *M* potassium phosphate buffer at pH 6.5. The ferredoxin was then eluted with 0.15 *M* Tris HCl buffer, pH 7.3, containing 0.67 *M* sodium chloride[3] in a volume of 120 ml.

First G-25 Sephadex column: Sixty ml aliquots of the eluate obtained in the previous step were desalted on a column of G-25 Sephadex (2.8 × 38 cm) with water. Ferredoxin was collected as a single black fraction following the elution of a contaminating reddish-brown protein.

Second DEAE-cellulose column: This step was based on a procedure reported by Tagawa and Arnon.[3] The desalted ferredoxin in a volume of 125 ml obtained in the previous step was applied to a DEAE-cellulose column (2.8 × 24 cm) equilibrated with 0.15 *M* Tris HCl buffer, pH 7.3, containing 0.15 *M* sodium chloride. The black ferredoxin fraction was eluted with this same buffer directly following an inactive reddish-brown protein fraction.

Second G-25 Sephadex column: The previous fraction was desalted by passage through another column of G-25 Sephadex as described above. The aqueous solution of ferredoxin was then lyophilized and dissolved in water to a volume of 22.5 ml.

In the following steps, crystallization was effected by adding solid ammonium sulfate to the

extract while continuously mixing with a magnetic stirrer. Precipitates were removed by centrifugation for 10 min at 39,100 × *g*.

First crystallization: Ammonium sulfate was slowly added to the extract to 60% saturation. Brown ferredoxin crystals were observed microscopically at this concentration of ammonium sulfate, but they were associated with a large amount of colorless, noncrystalline material. The precipitate was removed by centrifugation and discarded. Ammonium sulfate was added to the supernatant solution to 69% saturation. The crystals were collected by centrifugation, washed three times with 75% saturated ammonium sulfate solution, and dissolved in water to a volume of 11.5 ml. This solution was dialyzed 2½ hr against 15 liters of water; 18 ml of ferredoxin solution were recovered.

Second crystallization: Ammonium sulfate was added to the dialyzed solution to 65% saturation and the crystals were collected by centrifugation, washed twice with 70% saturated ammonium sulfate solution, and dissolved in water to a volume of 8.5 ml.

Third crystallization: Because ferredoxin was partially lost on dialysis, the ammonium sulfate was not completely removed prior to subsequent crystallizations; after dissolving the crystals in water, the residual ammonium sulfate concentration was estimated by conductivity measurements. For the third crystallization, the crystals forming between 55% and 60% ammonium sulfate saturation were collected by centrifugation, washed with 65% saturated ammonium sulfate solution, and dissolved in water to a volume of 2.7 ml.

Fourth and fifth crystallizations: For both of these steps, the crystals forming at 60% ammonium sulfate saturation were collected by centrifugation, washed with 65% saturated ammonium sulfate solution and dissolved in water to respective volumes of 2.5 and 1.9 ml. With certain preparations, the odor of hydrogen sulfide was detected after dissolving freshly prepared crystals in water. This was accompanied by a decrease in the specific activity and a loss of absorbancy at 390 mμ. It was found necessary to add buffer (final concentration of 0.05–0.10 *M* Tris HCl, pH 7.3) to stabilize these preparations during storage at 2°.

A summary of the purification and yield in each of these steps for *C. acidi-urici* ferredoxin is shown in Table 1. *C. pasteurianum* ferredoxin was isolated and crystallized by a similar procedure. *C. butyricum* ferredoxin was not crystallized but was purified about 400-fold by essentially the same procedure and by G-100 Sephadex and DEAE-cellulose chromatography. In the latter step, the column was eluted with a linear phosphate gradient essentially as described by Valentine, Jackson, and Wolfe.[2] The specific activity of this final 70% ammonium supernatant was about the same as that of crystalline preparations from *C. acidi-urici* and *C. pasteurianum* (see Table 3).

TABLE 1

PURIFICATION AND CRYSTALLIZATION OF *C. acidi-urici* FERREDOXIN

	Total protein mg	Total activity (units)	Yield %	Specific activity units/mg	390/280 mμ absorbancy ratio	390 mμ absorbancy per mg protein
Initial extract	25,600	21,280	100	0.8	—	—
First DEAE-cellulose column	2,136	17,544	82	8.2	0.08	—
First G-25 Sephadex column	763	11,675	55	15.3	0.14	—
Second DEAE cellulose column	430	11,575	54	26.9	0.21	—
Second G-25 Sephadex column	357	11,390	54	31.9	0.23	—
Second G-25 Sephadex column after lyophilization	342	10,049	47	29.4	0.27	—
First crystallization	274	11,845	56	43.3	0.56	2.7
First crystallization after dialysis	241	10,260	48	42.5	0.53	2.6
Second crystallization	189	8,585	40	45.5	0.63	3.1
Third crystallization*	71	3,645	17	51.1	0.77	3.5
Fourth crystallization	61	3,300	16	54.1	0.78	3.7
Fifth crystallization	57	2,997	14	52.9	0.78	3.7

* A significant portion of the total activity was accidentally lost at this step.

A photograph of the recrystallized protein from *C. acidi-urici* is shown in Figure 1. Crystals appeared as fine brown needles or rosettes. The corresponding crystals from *C. pasteurianum* were also brown but appeared to be more dense. Crystals from either source formed very dark brown or black solutions.

Results.—Occurrence of ferredoxin: Table 2 shows the relative ferredoxin content of certain bacteria as determined with the pyruvate clastic system of *C. pasteurianum*. Of the organisms tested, *C. tetanomorphum* contained the highest concentration of ferredoxin per gram of dry weight. Relatively large amounts were also found in *M. omelianskii*, *C. acidi-urici*, *C. cylindrosporum*, and *C. pasteurianum*, but *C. kluyveri* and *C. butyricum* contained considerably less. Ferredoxin could not be detected in anaerobically grown *E. coli*, *L. delbrueckii*, aerobically grown *E. coli*, *A. aerogenes*, either of two *Pseudomonas* species, or beef liver.

Spectral studies: The ultraviolet absorption spectra of *C. acidi-urici* ferredoxin was determined after each of five successive crystallizations. Extraneous material absorbing at 260 mμ was present in appreciable amounts after the first and second crystallizations, but this was eliminated after the third crystallization and the spectrum was then reproducible. The absorption spectra of *C. pasteurianum* ferredoxin was determined after each of four successive crystallizations. The absorption maximum at 280 mμ initially observed was reduced upon successive recrystallization until the peaks at 280 and 300 mμ reached the same value.

The comparative absorption spectra of five times crystallized *C. acidi-urici*, four times crystallized *C. pasteurianum*, and highly purified but not crystalline *C. butyricum* ferredoxins are presented in Figure 2. These spectra were similar in

FIG. 1.—Photomicrograph of recrystallized ferredoxin from *C. acidi-urici*.

TABLE 2

FERREDOXIN CONTENT OF SOME BACTERIA AND BEEF LIVER

Source*	ATCC no.	Units per gm dry cells	Units per mg protein in extract
Clostridium tetanomorphum	—	154	0.4
Methanobacillus omelianskii	—	92	1.1
Clostridium acidi-urici	—	90	0.3
Clostridium cylindrosporum	7905	71	0.2
Clostridium pasteurianum	6013	61	0.8
Clostridium kluyveri†	12489	32	0.1
Clostridium butyricum	6014	19	0.2
Escherichia coli	11097	0	0.0
Lactobacillus delbrueckii	—	0	0.0
Aerobacter aerogenes	—	0	0.0
Pseudomonas Sp.	11150	0	0.0
Pseudomonas P_2^{15}	—	0	0.0
Beef liver	—	0	0.0

* *A. aerogenes* and the *Pseudomonas* species were grown aerobically. *E. coli* was grown both aerobically and anaerobically. Bacterial extracts were prepared by subjecting from 250–1000 mg of lyophilized cells to a Raytheon 10 kc sonic oscillator for 30 min. Beef liver extract was obtained from 35 gm of fresh liver slices with a Potter-Elvehjem homogenizer. The extract was applied to a column (1 × 5 cm) of DEAE-cellulose and eluted as described under *First DEAE-cellulose column* of the section on *Materials and Methods*, and the eluate was analyzed for ferredoxin.

† Cells grown and dried in 1951 by Professor H. A. Barker.

that each showed a major absorbancy peak at 390 mμ, but differences were found in the ultraviolet region. The pronounced absorbancy peak at 280 mμ observed with ferredoxin from *C. acidi-urici* was significantly lower with ferredoxin from *C. pasteurianum* and was absent with that from *C. butyricum*. The shoulder observed at 300 mμ with *C. acidi-urici* ferredoxin appeared as a major ultraviolet absorbancy peak with *C. pasteurianum* and *C. butyricum* preparations. These differences in spectra may reflect the differences in chemical composition among the compounds; however, they may also be a reflection of the partial degradation of the proteins due to their variable stability. The ratios of the absorbancies at 280/390 mμ were 0.78, 0.81, and 0.77 and the ratios of the absorbancies at 280/260 mμ were 1.1, 1.2, and 1.1 for *C. acidi-urici*, *C. pasteurianum*, and *C. butyricum* ferredoxins, respectively. The spectra shown were measured with recently purified or crystallized preparations, for as previously mentioned, solutions of crystalline ferredoxin lost both 390 mμ absorbancy and enzymic activity when stored at 2°.

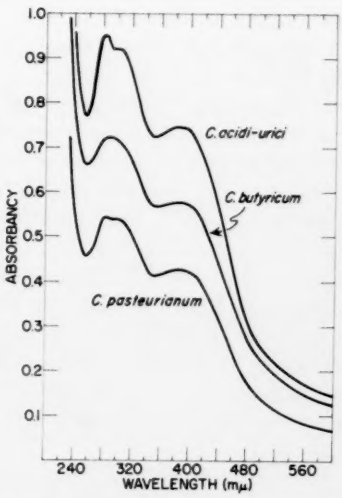

FIG. 2.—Comparative absorption spectra of clostridial ferredoxins. *C. acidi-urici* and *C. pasteurianum* ferredoxins were 5 times and 4 times crystallized, respectively. *C. butyricum* ferredoxin was purified 400-fold as given under *Materials and Methods*, but was not crystalline. The cuvettes contained 0.195, 0.141, and 0.154 mg ferredoxin/ml from *C. acidi-urici*, *C. pasteurianum*, and *C. butyricum*, respectively, in 0.07 *M* Tris, HCl buffer at pH 7.3. Spectra were measured with a Cary 14 *M* spectrophotometer.

Because of these differences in the ultraviolet absorption spectra, crystalline ferredoxin preparations from *C. acidi-urici* and *C. pasteurianum* were compared by infrared spectroscopy. The results presented in Figure 3 show that while there were great similarities between the infrared spectra of these ferredoxins, the notice-

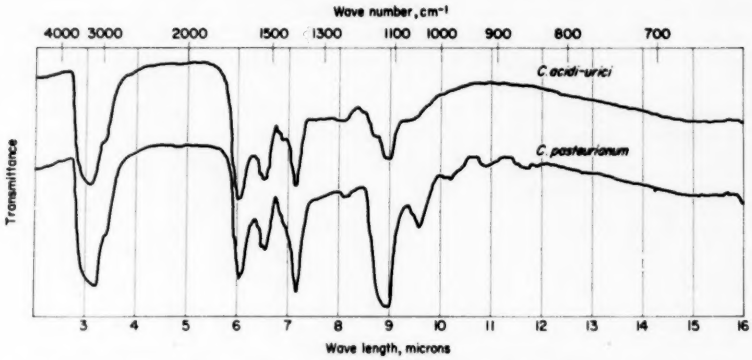
Wave number, cm⁻¹
4000 3000 2000 1500 1300 1100 1000 900 800 700
C. acidi-urici
C. pasteurianum
Transmittance
3 4 5 6 7 8 9 10 11 12 13 14 15 16
Wave length, microns

FIG. 3.—Infrared spectra of *C. acidi-urici* and *C. pasteurianum* ferredoxins. Spectra were measured with KBr pellets containing approximately 2 mg ferredoxin in a Baird-Atomic infrared spectrophotometer. *C. acidi-urici* and *C. pasteurianum* were 5 and 4 times crystallized, respectively.

able peak at about 1050 cm^{-1} found with *C. pasteurianum* was not present in the *C. acidi-urici* protein.

Iron and labile sulfide: Crystalline ferredoxin from either *C. acidi-urici* or *C. pasteurianum* contained considerable amounts of iron and sulfide loosely bound to the enzyme as shown in Table 3. Acidification of the protein caused rapid release of iron and H_2S; these factors were also rapidly dissociated at pH 13 as indicated by the decrease of the visible absorption at this pH. The *o*-phenanthroline method of iron analysis[12] permits the differential measurement of Fe^{++} and Fe^{+++} under some conditions. In the presence of protein solutions, however, Fe^{+++} is rapidly reduced to Fe^{++}. Analysis of ferredoxin by this method indicated that the major portion of the iron was present as Fe^{++} and that the small portion of the iron present as Fe^{+++} was rapidly converted to Fe^{++} during the analysis. Ferric iron when added to ferredoxin solutions in the presence of *o*-phenanthroline was also rapidly reduced. This method, therefore, did not permit the experimental determination of the oxidation level of iron in ferredoxin; however, it did permit the determination of the total iron content. The methylene blue method[13] which measures $S^=$ specifically indicated that ferredoxin contained approximately equivalent amounts of iron and sulfide. "Photosynthetic pyridine nucleotide reductase" also contains approximately two μatoms each of iron and free sulfide per molecule.[16]

Amino acid analysis: The amino acid composition of ferredoxin is shown in

TABLE 3

IRON AND LABILE SULFIDE CONTENT OF CLOSTRIDIAL FERREDOXINS

Organism	Iron per μmole protein*	"Labile sulfide" per μmole protein*	Specific activity units per mg
C. pasteurianum	9.6	10.9	51
C. acidi-urici	11.3	8.5	58
C. butyricum	10.7	6.1†	50

* Calculated on the basis of a molecular weight of 12,000 as indicated by a single ultracentrifuge determination.

† Sulfide was determined several weeks after purification probably accounting for the lower amount observed.

Analyses were done on four times crystallized *C. pasteurianum* ferredoxin, seven times crystallized *C. acidi-urici* ferredoxin, and highly purified *C. butyricum* ferredoxin.

Table 4. Ferredoxin from either clostridial species contains no histidine, methionine, leucine, or tryptophan. *C. pasteurianum* ferredoxin contains no arginine, whereas lysine and phenylalanine are absent from *C. acidi-urici* ferredoxin. In addition there are slight differences in serine, alanine, and proline content of the ferredoxin isolated from these two sources, while the remainder of the residues appear to be the same in both proteins. The generally slightly higher values for the μmoles of amino acid per gram protein obtained with *C. acidi-urici* ferredoxin is probably due to the fact that this sample was dried at 100° over P_2O_5, whereas the *C. pasteurianum* sample was dried by prolonged lyophilization. The proposed number of residues per mole, however, will remain uncertain until additional analyses are completed.

TABLE 4

AMINO ACID ANALYSIS OF FERREDOXIN FROM *C. pasteurianum* AND *C. acidi-urici*

Amino acid residue	μm/gm Protein*		Residues per mole§	
	C. pasteurianum	*C. acidi-urici*	*C. pasteurianum*	*C. acidi-urici*
Lysine	122	28	2	0
Histidine	6	10	0	0
NH_3†	1314	1447	20	20
Arginine	6	134	0	2
Cysteic acid	0	159		
Aspartate	1000	1108	16	16
Threonine	94	134	2	2
Serine	486	372	8	6
Glutamate	512	578	8	8
Proline	394	557	6	8
Glycine	482	566	8	8
Alanine	1000	1241	16	18
½ Cystine	505	332	8	8‡
Valine	692	742	12	12
Methionine	3	8	0	0
Isoleucine	562	622	10	10
Leucine	3	24	0	0
Tyrosine	120	169	2	2
Phenylalanine	119	18	2	0
Tryptophan	20	10	0	0
TOTAL			120	120

* These values are based on the weight of a four times crystallized preparation of *C. pasteurianum* dried by prolonged lyophilization and a five times crystallized preparation of *C. acidi-urici* dried to constant weight at 100°C over P_2O_5.

† The ammonia value given does not necessarily represent amide nitrogen.

‡ Cysteic acid included as ½ cystine in the proposed number of residues.

§ These calculations are based on a molecular weight of 12,000 and are therefore subject to further correction when more accurate molecular weight determinations are available.

Discussion.—Recent studies have shown that ferredoxin isolated from *C. pasteurianum* and spinach are interchangeable in mediating electron transfer in enzymic systems.[3] We have examined a number of other sources for ferredoxin and have found it present in all clostridial strains examined and in obligately anaerobic bacteria, but not in aerobic or facultatively anaerobic organisms. The ferredoxin from different sources is active in the pyruvate "clastic" system of *C. pasteurianum* as well as in the assay system derived from *C. butyricum*.

This protein has been crystallized from two clostridia having markedly different catabolic pathways. *C. pasteurianum* is a saccharolytic organism and can use nitrogen gas as its sole nitrogen source, while *C. acidi-urici* is a relatively fastidious organism which grows only on purines. The striking similarities between their ferredoxins is therefore worth noting. Both proteins have similar specific activities and similar iron and "labile sulfide" concentrations. However, differences between

the proteins are indicated by the ultraviolet and infrared spectral data. The amino acid analyses show that the ferredoxins of these organisms differ although the total number of amino acid residues are similar. Lysine is the only basic amino acid in *C. pasteurianum* ferredoxin, whereas arginine is the only basic amino acid in *C. acidi-urici* ferredoxin. The *C. acidi-urici* ferredoxin differs further by being free of phenylalanine. Both proteins are characterized by the mutual absence of histidine, methionine, leucine, and tryptophan.

The ferredoxins tested contain approximately equimolar amounts of iron and "labile sulfide" which, on the basis of their apparent simultaneous removal at extremely high or low pH values, appear to be closely associated. However, the relationship of this apparent association to the enzymic activity remains to be determined.

Summary.—Ferredoxin has been found in *C. tetanomorphum*, *M. omelianskii-C. acidi-urici*, *C. cylindrosporum*, *C. pasteurianum*, *C. kluyveri*, and *C. butyricum*. It has not been found in aerobic or facultatively anaerobic microorganisms or in beef liver. Ferredoxin has been crystallized from *C. acidi-urici* and *C. pasteurianum;* it has been purified about 400-fold from *C. butyricum* but has not been crystallized from this source. A method is described for the purification and crystallization of the protein from *C. acidi-urici*.

Purified preparations from *C. acidi-urici*, *C. pasteurianum*, and *C. butyricum* have essentially the same enzymic activity, an absorption maximum at 390 mμ, and approximately 1 μmole of "labile sulfide" and non-heme iron per mg. These preparations differ in their ultraviolet and infrared absorption spectra and amino acid composition. Crystalline preparations obtained from *C. acidi-urici* and *C. pasteurianum* lack methionine, histidine, tryptophan, and leucine, and contain only a single basic amino acid. *C. acidi-urici* ferredoxin contains arginine and no lysine, whereas *C. pasteurianum* ferredoxin contains lysine and no arginine. *C. acidi-urici* ferredoxin also lacks phenylalanine.

We wish to thank Professor F. H. Carpenter for use of the amino acid analyzer and Mr. H. Blair and Mr. P. Magee for generous supplies of *C. tetanomorphum* and *Pseudomonas* P_2. The technical assistance of Mrs. Shirley MacDonald is also gratefully acknowledged.

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*CONSERVATION OF A VIRAL RNA GENOME DURING REPLICATION AND TRANSLATION**

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In organisms with a DNA genome three principle modes of information transfer are recognized and distinguished by the end purposes they serve. The first is duplication, designed to provide exact copies for hereditary transmission. The second is a transcription which converts the information into RNA complementary copies. Finally, we have a translation from the four-unit language of the nucleic acids to the twenty-element parlance of the proteins. The existence of viruses with RNA genomes raises obvious questions of the mechanisms employed to attain the same ends.

It has recently been shown[1] that neither before nor after infection can one detect sequences in the DNA of the host cell which are complementary to that of the viral RNA. These results suggest that the RNA viruses do not employ DNA as an informational component at any step unique to the production of mature virus particles. One is led therefore to predict a mechanism of RNA synthesis involving an RNA dependent polymerase. The available evidence makes it unlikely that an enzyme of this sort pre-exists in the uninfected cell. All recognized RNA components, including "informational,"[2, 3] ribosomal,[4, 5] and transfer RNA[6, 7] have been shown to be complementary to sequences in homologous DNA. Their formation can therefore be adequately explained in terms of the DNA-dependent RNA polymerase.[8, 9] This conclusion is further strengthened by the observation that actinomycin *D* inhibits normal cellular RNA synthesis but is unable to interfere with viral RNA formation.[10, 11]

Considerations such as these lead to the prediction that the viral RNA injected into the host cell must contain the structural program for a new RNA polymerase. Since this enzyme must be synthesized *before* replication can begin, it follows that the entering RNA must *itself serve as a protein program and be conserved during its translation into protein.*

It is the purpose of the present paper to provide information pertinent to the prediction of conservation. For reasons which will become apparent it was technically simpler to answer the following more inclusive question: *Is the incoming strand conserved during translation and replication?* To identify the parental RNA, double labeling with N^{15} and P^{32} was used. Recovery of the original doubly labeled strand can be interpreted unambiguously as conservation only if the following possible complications can be eliminated: (*a*) the existence of unattached or reversibly at-

tached virus particles; (*b*) contamination of the sample analyzed with infected cells which did not produce virus components from the injected strands; (*c*) the presence of nonparticipating strands in cells which were multiply infected. Procedures will be described which avoid these sources of confusion. The data obtained are consistent with complete conservation of the RNA genome during all the replications and translations required for a lytic cycle.

Materials and Methods.—(a) *Cells, virus, and media:* The bacterial virus MSϕ2 was provided by Dr. Alvin J. Clark and is similar to the RNA bacteriophage (f2) described by Loeb and Zinder.[12] The phage was grown and assayed according to the procedures of these authors. Infectious centers were, as usual, assayed in the presence of MSϕ2-antiserum. The minimal medium (SC) routinely used is the same as that employed previously.[2] The general buffer used, designated by TM, is Tris at 0.01 *M* and 0.005 *M* Mg^{++} buffered at pH 7.4.

(b) *Preparation of extracts from infected cells:* The lysozyme-freeze thaw method as detailed by Hayashi and Spiegelman[2] was used to prepare extracts which were cleared by centrifugation at 15 *g* for 15 min to yield a supernatant (15G-15S). Where pertinent these were analyzed in linear sucrose gradients for distribution of $O.D._{260}$ and radioactivity. In all cases acid precipitable radioactivity was assayed on millipore membranes in a Packard scintillation spectrometer as detailed previously.[2] *Ribonucleic acid preparation and purification:* All RNA preparations, from either mature phage or cell lysate, were purified by the phenol procedure.[13] *E. coli* 23S RNA-H^3 was prepared by the method of Yankofsky and Spiegelman.[4]

(c) *Cesium sulfate density gradient centrifugation:* The cesium sulfate was obtained from Penn Rare Metals, Inc., Revere, Pennsylvania, and recrystallized repeatedly from water until the optical density at 260 mμ of a 59% (w/w) solution was less than 0.1. The densities of ribonucleic acids in cesium sulfate were determined by the method of Hearst and Vinograd.[14] For centrifugation in the preparative Spinco Model L ultracentrifuge, 2.1 ml of a cesium sulfate solution (ρ = 1.88), RNA solutions and 0.01 *M* phosphate buffer, pH 6.8, were mixed to a final volume of 3.0 ml. The final density of the solution was 1.617. The samples were centrifuged for 72 hr at 33,000 rpm at 25°C in the SW 39 rotor. Fractions of 0.061 ml were then collected from the bottom of the tubes. The refractive index of every fifth sample was determined to convert to density by means of a standard curve. The samples were then diluted and analyzed for $O.D._{260}$ and radioactivity.

(d) *Preparation of N^{15}-P^{32}-labeled virus:* The growth medium contained: 0.055 *M* $N^{15}H_4Cl$; 0.170 *M* NaCl; 0.054 *M* KCl; 0.006 *M* $MgSO_4$; 0.0005 *M* PO_4; 0.0006 *M* $FeCl_3$; 0.050 *M* Tris buffer, pH 7.2, 0.011 *M* glucose, 0.0002 *M* $CaCl_2$, 23.5 mc P^{32}.

E. coli K-10 was added to 100 ml of medium to give an initial cell density of about 7×10^7 per ml and when it reached 2×10^8, P^{32} was added. At 5×10^8, the cells were infected at a multiplicity of 0.1 with MSϕ2. The culture was allowed to shake for 12 hr after infection.

At this time the cell suspension was centrifuged to separate the unlysed cells from the lysate. The unlysed cells were suspended in 3.0 ml of TM buffer containing 300 μgm of lysozyme per ml and 15 μgm of DNAase per ml. The suspension was kept at room temperature for 30 min at which time 0.5 ml of chloroform was added. This mixture was vigorously agitated for 15 min and then centrifuged to remove cell debris. The supernatant was combined with the original lysate supernate.

The combined supernate was made 2.0 *M* in ammonium sulfate and kept in the cold (4°C) for 3 hr. The phage which precipitates was removed by centrifugation at 10,000 rpm for 10 min. The phage was then suspended in 2.0 ml of TM buffer and dialyzed against TM buffer for 5 hr. After dialysis, the phage was layered on a 3–20% linear sucrose gradient containing 10^{-4} *M* $MgCl_2$ and centrifuged for 4 hr at 25,000 rpm at 5°C in an SW 25.1 rotor of the Spinco Model L. At the end of this time, the bottom of the swinging bucket tube was pierced and 1.0 ml fractions were collected and analyzed for radioactivity and plaque forming ability. The peak regions were collected and dialyzed against 3 changes of 0.01 *M* phosphate buffer. The phage preparation after dialysis was analyzed for optical density at 260 mμ, plaque forming units and radioactivity.

(e) *Details of a conservation experiment: E. coli* K-10 cells were grown to a density of 5×10^8 cells per ml in 200 ml of SC medium at 37°C. They were washed twice with SC medium minus

glucose and finally resuspended in 10 ml of SC medium minus glucose. The $N^{15}P^{32}$ labeled MSϕ2 phage was then added to the cell suspension and the mixture was maintained at 37°C for 30 min without aeration.

After 30 min of adsorption 90 ml of pre-warmed complete SC medium was gently added to the phage-cell mixture. This is considered zero time of infection. After 1 min aeration by shaking was instituted. At 10 min the culture was chilled by swirling in an ice bath and then centrifuged at 6,000 rpm for 5 min. The sedimented cells were then washed two times with cold, glucose-free, SC medium containing 0.01 *M* versene. The wash medium was kept for analysis of removed radioactive phage. The infected cells were finally resuspended in pre-warmed complete SC medium. At this time, aliquots for infectious centers were taken. The culture was again shaken until 30 min had elapsed at which time bentonite was added to a final concentration of 5 mg per ml. The culture was then shaken for 30 more min. At this time the culture was again chilled. The suspension was centrifuged at 12,000 rpm for 15 min to separate the unlysed cells from the lysate. The cell pellet was analyzed for its radioactive content. The supernatant fraction was analyzed for radioactivity and was made 0.01 *M* in versene to chelate the Ca^{++} present which otherwise formed an insoluble precipitate upon the addition of EtOH. Then 10 ml of 2.0 *M* potassium acetate and 220 ml of 100% ethanol was added to precipitate the nucleic acids in the supernate. The precipitate was collected by centrifugation at 10,000 rpm for 10 min and resuspended in 3 ml of TM buffer. To this solution was added 3.0 ml of water-saturated phenol and the mixture was vigorously agitated at room temperature for 15 min. The phenol and aqueous layers were separated by centrifugation at 6,000 rpm for 5 min. The aqueous layer was removed and extracted twice with 3 volumes of ether (anhydrous). The ether was removed by blowing nitrogen through the solution. Finally, the solution was made 0.2 *M* in potassium acetate and 2 volumes of 100% ethanol was added to precipitate the nucleic acid. The precipitate was resuspended in 2.0 ml of TM buffer and used for density gradient centrifugation in Cs_2SO_4. Final recovery of purified RNA from the lysate was between 60–70%.

Results.—(a) *Identification of parental strands in a population of RNA molecules:* An answer to the question of conservation requires the identification of the injected strand in a mixture of progeny and cellular RNA components. Obviously, the parental RNA would be present at levels precluding their identification as optically observable components. Consequently, two identifying isotopic labels were used, N^{15} to provide a unique position in a density gradient, and P^{32} to permit detection of the original strands.

Preliminary reconstruction experiments were carried out to see how readily N^{15}-P^{32}-labeled phage RNA could be identified in the presence of its unlabeled counterpart and ribosomal RNA. A difficulty was encountered due to a tendency of phage RNA to aggregate with ribosomal RNA, if the latter is present in excess. This complication was avoided by preparing P^{32}-viral RNA of high specific activity, permitting the use of small aliquots, and adding unlabeled carrier viral RNA in excess to displace the labeled RNA from any existent complex with ribosomal RNA.

The separation of tritiated ribosomal RNA from unlabeled virus RNA in a cesium sulfate gradient is shown in Figure 1*A*. In this case the amount of ribosomal RNA added is small and its position is located by its H^3 label. Figure 1*B* demonstrates that N^{15}-P^{32}-labeled virus RNA can be easily distinguished from unlabeled viral RNA. The amount of N^{15}-P^{32}-RNA added was deliberately kept low in the reconstruction experiment in order to conform to the conditions expected in an actual conservation experiment. Evidently, aggregation between the N^{15}-P^{32} and the unlabeled RNA does not occur. It is clear from Figure 1 that density gradient centrifugation permits a ready identification of each type of RNA in a mixture, providing radioactive labeling is used in addition as an aid to location.

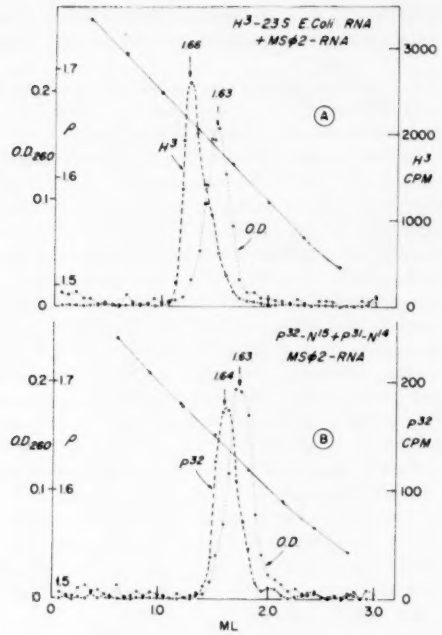

FIG. 1.—*Fractionation of RNA by cesium sulfate density gradient centrifugation:* (*A*) The cesium sulfate solution contained *E. coli* 23S RNA-H^3 (14 μgm, 13,000 cpm) and MSφ2 RNA-$N^{14}P^{31}$ (50 μgm) in a final volume of 3.0 ml with a density of 1.612. The sample was centrifuged for 72 hr at 33,000 rpm at 25°C in the SW39 rotor of the Spinco Model L ultra-centrifuge. At the end, fractions of 0.061 ml were collected from the bottom of the tubes, diluted and analyzed for optical density at 260 mμ and radioactivity as described in *Methods*. The straight line gives the density gradient. (*B*) The cesium sulfate solution contained MSφ2 RNA-$N^{15}P^{32}$ (0.05 μgm, 1200 cpm) and MSφ2 RNA-$N^{14}P^{31}$ (50 μgm) in a final volume of 3.0 ml with a density of 1.612. Other details as in (*A*).

(b) *Evidence for complete conservation of the parental strands:* To obviate the complications mentioned in the introduction, and permit a definitive decision, the following precautions were introduced into the conservation experiment: (1) Low multiplicities of infection (0.05 or less) were used to avoid the possibility of having active and inactive strands in the same cell. (2) Unattached or reversibly attached particles were eliminated by washing the cells prior to lysis. (3) To insure restriction of the examination to infected complexes actively producing phage components, analysis was confined to the RNA released after lysis.

Since the virus particles are labeled with P^{32}, the various steps can be readily monitored. It was found that two centrifugal washings with buffered versene effectively removed all unadsorbed or reversibly attached virus particles. The counts which remain with the cells after the washing permit an upper limit calculation of the actual multiplicity of infection. To satisfy the third criterion, incubation had to be continued until lysis occurred and unlysed cells removed prior to isolation of the RNA. This required protection of the released RNA from nucleolytic degradation in the crude lysate. Using labeled phage RNA in reconstructed systems, it was found that the addition of bentonite[15] permitted full recovery.

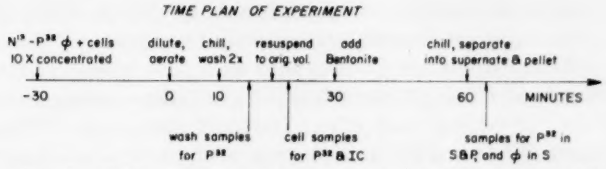

FIG. 2.—*Outline for the conservation experiment:* The times of additions, operations and removal of samples are indicated. The symbols employed denote the following: *IC*, infectious centers; *S*, lysate supernatant; *P*, lysate pellet; φ, phage count. See other details in *Methods*, section *e*.

As a result of these and other preliminary investigations a procedure for such experiments was finally evolved which yielded consistent results with satisfactory regularity. The time plan of such experiments is diagrammed in Figure 2 and a typical protocol detailed in *Methods* (section *e*).

Depending on the initial ratio of phage to bacteria, between 70–90% of the particles were lost in the wash fluids. The inputs of virus particles were accordingly adjusted to yield the finally desired multiplicities of infection. Table 1 summarizes a number of experiments carried out at different multiplicities. Recorded is the radioactive material which remains with the cells and is ultimately recovered as acid precipitable material after lysis has occurred. It will be noted that the per cent recovery of the fixed P^{32}-RNA is excellent, ranging from 95–98%. Further, between $^1/_3$ and $^1/_6$ is released into the supernatant fraction, the remainder being found in cells which have not lysed by 60 min. In addition, there is reasonably good agreement between the P^{32}-phage equivalents found in the lysate and the number of infectious centers assayed immediately after the washing and subsequent resuspension. The yield of active virus particles found per lysed cell in such experiments ranged from 400–900. This is somewhat lower than the 1,000–2,000 found in the same medium with cultures allowed to complete the lytic cycle undisturbed.

We now turn our attention to the P^{32}-labeled RNA found in the supernatant fractions of the lysates formed after 60 min. It may first be noted that in all cases it was found that this RNA is as sensitive to RNAase as added control RNA, suggesting that it is all free. Under the same conditions labeled RNA contained within intact virus particles is resistant to degradation. Of immediate interest is the question of whether the P^{32} is still in association with N^{15}. The RNA of the supernatant

TABLE 1

DISTRIBUTION OF P^{32} IN CONSERVATION EXPERIMENTS

Each experiment involved 100 ml of starting culture. In all cases the numbers represent total found. Conversion of counts to phage equivalents was done on the basis measuring the O.D. at 260 (1.10) and the cpm per ml (5.1×10^7) of the purified N^{15}-P^{32}-virus particles. On the basis of comparison of plaque formers in freshly purified phage with O.D. one finds that one O.D. is equivalent to ca. 1.7×10^{12} particles.[18] Using these numbers yields a value of 2.7×10^{-5} cpm/particle. The multiplicity of infection was calculated by dividing the total counts irreversibly fixed in the washed cells in phage equivalents by the total cell number.

Experiment	A		B		C		D	
Fractions	Counts	φ-equivalents	Counts	φ-equivalents	Counts	φ-equivalents	Counts	φ-equivalents
Washed cells	10,300	3.7×10^9	23,400	8.4×10^9	47,600	1.7×10^{10}	93,400	3.4×10^{10}
Lysate pellet	8,310	3.0×10^9	19,350	7.0×10^9	35,530	1.3×10^{10}	70,700	2.5×10^{10}
Lysate supernate	1,400	0.5×10^9	4,700	1.7×10^9	9,700	3.6×10^9	19,200	7.0×10^9
Per cent recovery	94	—	98	—	95	—	96	—
Infectious centers	—	1.1×10^9	—	2.0×10^9	—	3.6×10^9	—	8.6×10^9
Cell number	—	8.7×10^{10}	—	9.5×10^{10}	—	8.5×10^{10}	—	9.0×10^{10}
Multiplicity of infection	—	0.042	—	0.084	—	0.2	—	0.38

fraction was purified according to the procedure detailed in *Methods*. Recovery of the final product ranged between 60–70%. It was then mixed with carrier P^{31}-N^{14}-virus RNA and banded in Cs_2SO_4. A typical outcome is given in Figure 3. It is clear that virtually all of the P^{32} is still found in a strand possessing the density characteristic of the N^{15}-labeled viral RNA. Little, if any, of the P^{32} has found its way either into the ribosomal RNA density region or into that which corresponds to progeny viral RNA. The band width of the P^{32}-peak indicates that no breakdown leading to size dispersity has occurred. This was further confirmed by chromatography on columns of methylated albumin.[18] In a number of repetitive experiments involving multiplicities of infection ranging from 0.04 to 0.09, identical results were obtained.

Discussion.—In the experiments described, the RNA examined is obtained from the lysate which contains active particles corresponding to an average burst of about 600 per lysed cell. This would seem to insure that the radioactive strands have been derived from cells actively synthesizing the necessary viral RNA and protein components. At the effective multiplicity employed (about 0.05), no more than 5 per cent of the infected cells would be expected to contain more than one viral equivalent. Since virtually all the RNA is accounted for, and purification yields 60–70% of the starting material, it would seem safe to conclude that a fair sample of actively participating RNA has been subjected to analysis.

The fact that the P^{32} bands in the density gradient at a location characteristic of the N^{15}-RNA, and that no P^{32} is found at densities corresponding to either newly formed viral or ribosomal RNA, argues for complete conservation. We conclude that the original strands of an RNA virus can be recovered intact at the end of a complete lytic cycle.

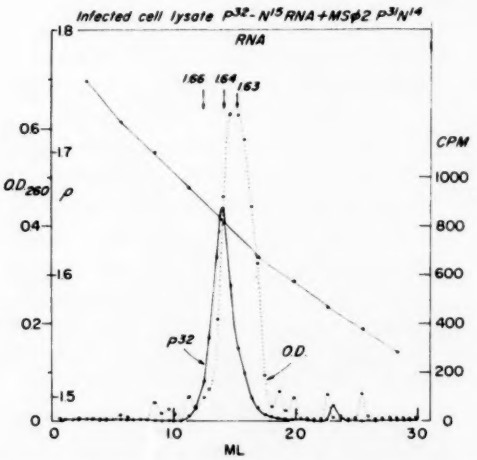

FIG. 3.—*Identification of components in lysate RNA by density gradient centrifugation:* The cesium sulfate solution contained the phenol purified lysate RNA (5,280 cpm and a total optical density of 0.5 at 260 mμ) and 200 μgm of marker MSφ2 RNA-$N^{14}P^{31}$ in a total volume of 3.0 ml at a density of 1.612. Other details as in legend for Figure 1 (*A*) and in section *e* of *Methods*.

It may be noted, in passing, that examination of the RNA in cells which have failed to lyse reveals that, in at least a portion, breakdown of the injected strand has occurred.[16] This further emphasizes the necessity of focusing attention on those cells which can produce virus particles in any attempts at understanding the ultimate fate of the incoming viral RNA.

The fact that all the parental RNA found in the lysates is RNAase sensitive suggests that the input strands are excluded from the final maturation process. This result is consistent with the finding of Davis and Sinsheimer[17] who, on direct examination of the mature virus yield, failed to detect original parental strands.

While the experiments summarized answer the question posed, they raise a number of other issues for experimental resolution. As pointed out in the introduction, there are good reasons for presuming that the incoming strand must serve as a structural program for a new protein. While the possibility of new RNA synthesis was not completely eliminated, the recent studies[20, 21] on *in vitro* synthesis of viral proteins on addition of homologous viral RNA strongly suggest the same conclusion. Further, we have shown in results to be detailed elsewhere[18] that the injected strand does indeed behave like a message. It is found in association with 80S ribosomes from the very onset and remains there throughout the course of the infection. The present experiments demonstrate, nevertheless, that they are conserved. However, indigenous genetic messages of these cells appear to be unstable and in continual turnover.[9, 10, 19] We are faced with the problem of finding the mechanism which permits destruction of one message and not another. Whatever the outcome, it is clear that instability is not a necessary property of all RNA molecules which serve as translatable messages for protein synthesis.

The problems of replication and transcription of RNA genomes are especially intriguing since they can still provide us with an interesting deviation from the expected. In particular, it should be noted that DNA genomes *must* transcribe their information into complementary RNA copies for use in protein synthesis. On the other hand, we have seen that RNA genomes are already translatable messages. Consequently, complementary transcription is not only unnecessary but is indeed likely to result in the formation of a nonsense strand, useless for protein synthesis. Thus, if complementary copying occurs it would be employed only for replicative purposes. However, replication can, in principle, occur via identical copies by readily designable mechanisms. The possibiiity must, therefore, be entertained that we may yet find that the RNA viruses have completely bypassed the use of complementarity.

Summary.—In an attempt to get an answer to the question of conservation, the RNA of mature virus particles was doubly labeled by growth in the presence of N^{15} and P^{32}. The RNA recovered after completion of lysis was banded in gradients of Cs_2SO_4. The two isotopes were recovered in the same RNA strands, corresponding in density to the N^{15}-RNA originally injected. The experiments were carried out under conditions which avoided the ambiguity which would be generated by the presence of nonparticipating strands or inactive virus particles.

The data are consistent with the conclusion that the parental strands of an RNA virus are completely conserved during all the replications and translations required to produce a full yield of mature virus particles. Since it is very likely that the incoming viral RNA must serve as a genetic message, the results indicate that in-

stability is not a mandatory attribute of RNA molecules which serve as programs for protein synthesis.

* This investigation was aided by grants in aid from the U.S. Public Health Service and the National Science Foundation.

† Postdoctoral Fellow, U.S. Public Health Service.

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AN APPROACH TO THE EVOLUTION OF METABOLISM

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The purpose of this communication is to raise some questions and offer specific suggestions concerning the general problem of how intermediary metabolism developed on this planet.

The speculations which follow are based upon a concept that there was a critical era in the evolutionary process several billion years ago at the period represented by the vortex in Figure 1 (a diagram suggested by a similar one used by N. W. Pirie[1]). It was at this time that there emerged a dominant colony of primitive cells which had the basic features (indicated below) that we find today in all living cells: there had been established the modern mechanisms for catalysis (enzymes) and for inheritance (nucleic acids); the basic pattern for cell structure (double lipid-protein membranes) had evolved; and by that time there had been established meta-

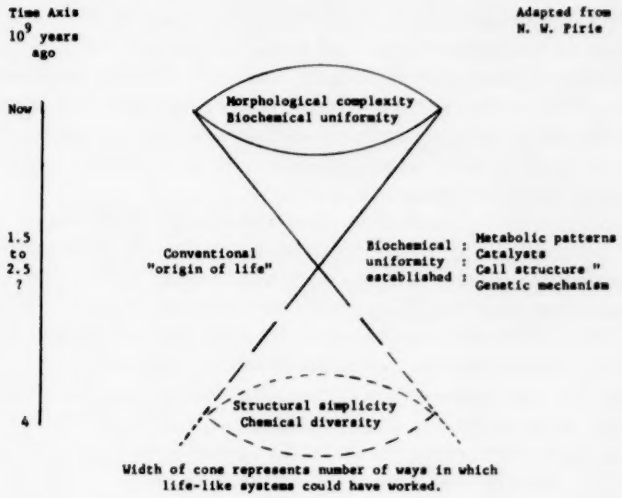
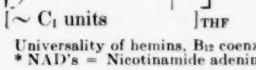


FIG. 1.

bolic systems functioning with *exactly* the same metabolites used by *every single modern cell*—the same set of L-amino acids, the same nucleotides, the same group of D-saccharides, the same constructions of lipid material, and, importantly from the standpoint of this presentation, the same array of organic cofactors.

Modern Organic Cofactors Showing *Units Transferred* and *Universal Carriers*

[~ P]$_{ADP}$	[~ acyl]$_{CoA}$
[(2H)]$_{NAD's^*\ and\ FN's}$	[~ CO_2]$_{Biotin\text{-}E}$
[~ aldehydes]$_{TPP}$	[~ saccharides]$_{UDP}$
	[~ lipid bases]$_{CDP}$
[~ C_1 units]$_{THF}$	[~ amino acids]$_{GDP}$(?)

Universality of hemins, B_{12} coenzymes, lipoic acid, undetermined.
* NAD's = Nicotinamide adenine dinucleotides formerly designated DPN and TPN.

Although the monomers in proteins and nucleic acids have remained unchanged since the time biochemical uniformity was established, polypeptides and polynucleotides must have been undergoing continual changes since the time of their first formation—just as they are today—changes making possible the evolution of modern cells of extreme diversity in structure and function. In contrast to proteins and nucleic acids, the organic cofactors—including molecules as complex as coenzyme A—must have remained immutable (down to the last atom) for two to three thousands of million years; and at some time previous to the emergence of the dominant colony, the types of chemical reactions which each of these ten to twelve cofactors catalyze must have been incorporated as absolutely essential components into the metabolism of the developing biological system which was eventually to populate this planet.

How did this uniform metabolic pattern that all living things possess evolve? What was the sequence? Did some chance polymerization of nucleotides (ran-

domly formed in a primordial soup) produce specific nucleic acids which triggered the formation of polypeptides, and did these polypeptides in turn have specific catalytic functions which enabled them to establish a uniform metabolic pattern? Such viewpoints have been explicitly stated on occasion and often implied. More reasonable hypotheses in which metabolism antedates enzymes and nucleoproteins have been set forth in broad generalities by several scientists—including Anker,[2] Bernal,[3] Calvin,[4] Gaffron,[5] Oparin,[6] and Pirie.[1]

Is it possible, today, to construct a *specific* scheme which would represent a reasonable pattern by which modern metabolic pathways could have evolved in some very primitive system? I believe such a pattern can best be formulated on an initial premise that there was in a primitive system a stepwise accretion of organic cofactors—these cofactors being the *functional* ancestors (not necessarily the structural ancestors) of the modern universal cofactors listed above; and, as a second premise, that these ancestral cofactors could and did function catalytically without proteins (just as it is possible to so demonstrate with most modern cofactors), and that these primitive organic cofactors (along with inorganic ions) acting on surfaces were the original "bio" catalysts, active long before specific polypeptides evolved. (During this speculation, it may help to visualize the postulated processes by using Bernal's hypothesis[3] that clays supplied the physical matrices and surfaces needed by systems in the very early stages of biochemical evolution.) As the developing system evolved, each of the earliest cofactors was replaced by a more complex, more efficient structure—a process that continued over and over until the full array of modern carriers had finally taken over at the chronological vortex in Figure 1.

The most fundamental type of biochemical reaction, one which is a prerequiste of all living cells, is the transformation of free energy from the environment into "high energy" reactive anhydrides of phosphoric acid. In modern cells, these reactive phosphate groups (~P) are accepted, transported, and donated by an ADP carrier system. Presumably much simpler, but analogous, mechanisms must have been one of the earliest acquisitions of the system which was to become dominant. Therefore, for the purpose of this discussion, let it be assumed that there was a functional ancestor of ADP (indicated as "ADP" in the accompanying figures) capable of accepting and donating reactive phosphate groups. Although there are countless reactions in modern cells using the energy of reactive phosphate groups, there are an extremely limited number of metabolic mechanisms known for transforming other types of free energy into this particular form. Of these, there is one reaction which comes closest to being universal. It also is by far the simplest from the standpoint of chemical mechanism, and hence it may be considered the most primitive of such modern reactions. It is the dehydration of glycerate-2-phosphate (glycerate-2-P), a simple molecule that undoubtedly was formed during the eons of chemical evolution. In this dehydration reaction a relatively unreactive, "low energy" phosphate ester is transformed into the "high energy," reactive anhydride of phosphoric acid and an unstable enol of pyruvic acid (Fig. 2*A*). With this reaction as a central nucleus, what subsequent events can occur in the same vicinity? A second molecule of glycerate-2-P, by reacting with the ~P generated from the first molecule, can be converted to glycerate-2,3-diphosphate (Fig. 2*B*). Interestingly, this compound is a necessary cofactor for the conversion of glycerate-3-P

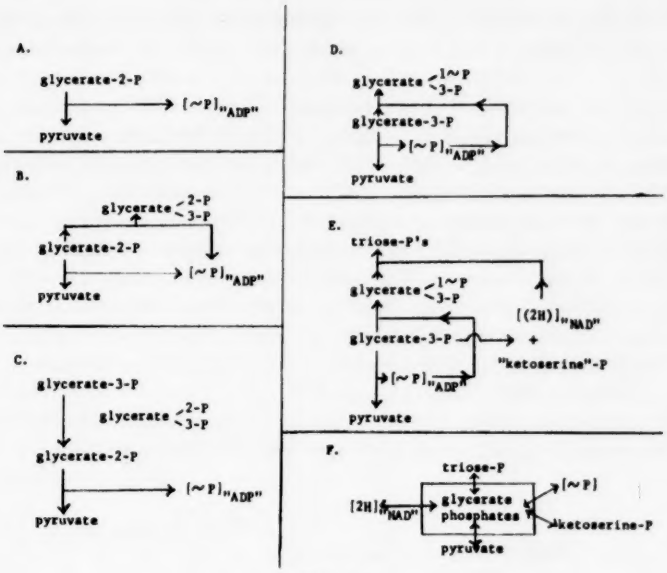
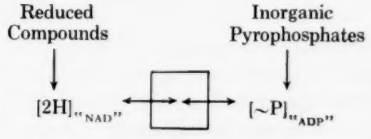


FIG. 2.

to glycerate-2-P. Hence, the product of a metabolite (the ~P) reacts with the metabolite itself (glycerate-2-P) to form a catalyst (glycerate-2,3-diphosphate) needed for the formation of more of the metabolite (Fig. 2*C*). (Presumably, the conditions which would lead to the chemical formation of the glycerate-2-P would produce equally as much or more of the isomeric glycerate-3-P, and primordial chromatography on the clay surfaces could be expected to further concentrate these two species of phosphate esters in the same localities.) Other molecules of glycerate-3-P can in turn serve as an acceptor for a ~P generated from glycerate-2-P, forming the highly reactive glycerate-1,3-diphosphate (Fig. 2*D*). The latter compound, however, by reacting with an ancestral hydrogen carrier ("NAD") which has been reduced by still another glycerate-3-phosphate, can form a triose-P, a compound in which the free energy has been trapped in a more stable configuration (Fig. 2*E*). Figure 2*F* summarizes the process of reactions centered around this core of four intimately related glycerate phosphates upon the acquisition of the two carriers. This is a summary of the "internal" exchanges. But what about the relationship of this closely knit system with its "external" environment?

The diagram below pictures how this primitive system (Fig. 2*F*) could make "nutritional" contact with energy sources of the environment.



This contact of course depends on the two carriers; with a hydrogen carrier the system could draw upon the H's in the reduced compounds in the "primordial

soup," and with a phosphate carrier the system could draw upon the energy in the inorganic pyrophosphates which presumably were abundant in the primitive environment.[7]

By subsequent accretion of other ancestors of modern cofactors, the glycerate phosphate core is further expanded (Fig. 3): (1) to the left of the core the NAD-like carrier establishes metabolic contact (*a*) with glycerol-P and (*b*) with the C_4 dicarboxylic acids, the latter being formed by a type reaction found in most modern organisms and currently catalyzed by the NADP(TPN)-dependent malic enzymes; (2) above the core an ancestral TPP, a coketolase, carries a reactive C_2-hydroxyaldehyde from hexose-P's (formed by aldol condensation from triose-P's, maybe with the catalytic aid of ferrous ions) to other triose-P's forming pentose-P's; (3) at the lower right of the core a B_6-like coenzyme is shown carrying amino groups from the environment to the three keto acids of the expanding core forming the corresponding α-amino acids; and (4) acquisition of a folic acid coenzyme ancestor ("THF") enables the system to convert serine to glycine, and, equally important, provides the reactive C_1 unit needed in subsequent metabolic evolution.

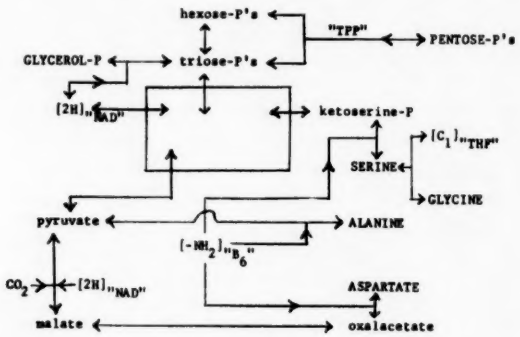


FIG. 3.

The establishment of metabolic contact by this system with glycerol-P, with these four amino acids, and with the dicarboxylic acids has two important implications: (1) when the environment is rich in these substances (as presumably it was in the earliest stages of biochemical evolution), then the system can feed on these particular compounds, using them as sources of carbon that can be anabolized by the reactions of the core; and (2) when the concentrations of these substances subsequently become low or play out, the system can then produce them metabolically. Also, examination of the right-hand margin of Figure 3 shows that such a system has the metabolic capacity for forming the essential intermediates—pentose-P, C_1 units, glycine, and aspartate—which at later stages of development will be needed for the biosynthesis of nucleotides. These considerations may have significance in attempting to resolve the question: Were the specific amino acids, the specific porphyrins, and the specific nucleotide bases found in modern cells "selected" because they were the particular compounds that occurred most abundantly in the pre-biotic environment and because some system "learned" how to make them when they disappeared from the environment, or did these compounds be-

come the foundation of life because their "bio" synthesis evolved directly from a central core in a system such as shown here?

To return to the question of the nature of these ancestral cofactors: Could not the triose phosphate have served as the ancestral "NAD"s, the triose-P being the oxidized, unloaded carrier, and glycerol phosphate being the reduced, loaded c r- rier (Fig. 4)? (This triose-P—glycerol-P system does in fact act as the transport system in the flight muscles of modern insects, the glycerol phosphate transporting H's from the cytoplasm to the mitochondria and returning unloaded as triose ph s- phate.[8]) Could it not be that the glycerate phosphate core, generating and accepting high energy phosphates, served as the initial ADP-like carrier system in the early stages of evolution? Similarly, could not the keto acid-amino acid pairs pictured in Figure 3 have served as the first functional form of our modern B_6 coenzymes, and could not compounds donating or accepting single carbon units have acted as the carriers in processes that today require tetrahydrofolate? Subsequently, could not more and more complicated molecules *formed from metabolism* (and functioning more effectively with evolving polypeptides) have taken over the specific "transporting" jobs?

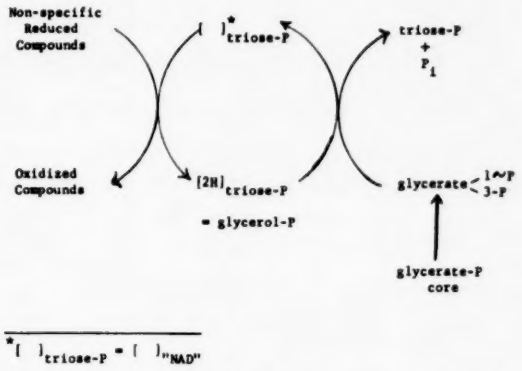


FIG. 4.

In Figure 4 a postulated catalyst, triose-P, is shown to be catalyzing its own synthesis—one molecule (without being consumed) acting to create autocatalytically a second molecule of itself, in other words, a replication process.

The possibility of such replication raises the last question to be considered in this discussion: Could not these metabolites that have been postulated as functioning as primitive cofactors also be considered as the original genetic material? *First*, they would be functioning autocatalytically in the expanding system described, causing (directly or indirectly) their own synthesis and accumulation; and *second*, because of this accumulation, when aggregates fragmented, the cofactors would be passed from the parental aggregates to the daughter fragments where they would initiate and maintain the types of reactions (and hence the metabolic sequences) that they had been directing in the parental system.

Speculation on the subsequent development of more precise genetic and regulating mechanisms should certainly take into account the fact that the ribonucleic

acids which evolved are polymers of the four nucleotides which were selected to transport units of energy (ADP) and to transport the monomers used in synthesizing carbohydrates (UDP), lipids (CDP), and proteins (GDP).

Summary.—By postulating a stepwise accretion of functional ancestors of modern cofactors, it is possible to construct a specific scheme of evolution of metabolism, the initial phase of which is very briefly sketched here, in complete agreement with modern biochemistry. In this scheme simple metabolites themselves served as these functional ancestors of modern cofactors. Because they in effect catalyzed their own replication, because they were passed from parental to daughter systems, and because they determined the metabolism of the daughter aggregates, they can be considered to constitute the primitive hereditary apparatus.

The author wishes to express appreciation to a number of his colleagues and students for many stimulating and provocative discussions concerning the origin of metabolism. He is especially indebted to Daniel M. Ziegler for invaluable advice and suggestions in the formulation of the premises presented.

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*AN UNUSUAL DNA EXTRACTED FROM BACTERIA INFECTED WITH PHAGE T2**

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Bacteria infected with phage T2 contain phage-precursor DNA, phage particles, and intermediate structures.[1] Chloramphenicol[2] and 5-methyltryptophan[5] inhibit protein synthesis and prevent formation of phage particles but do not directly inhibit DNA synthesis. The DNA that accumulates in the presence of these inhibitors presumably represents DNA in its replicating state.

This paper describes an attempt to characterize such DNA. It appears to consist, at least in part, of DNA structures that are larger than the molecules found in phage particles. Except for properties dependent on size, the two forms of phage DNA show similar characteristics.

Methods.—Intrabacterial DNA was isolated as follows. A culture of *Escherichia coli* strain H, grown to 5×10^8 cells/ml in synthetic medium, was infected at time 0 with about 5 particles of phage T2H per bacterium.[1] Ten min later, protein synthesis was stopped by adding 30 μg/ml

of chloramphenicol or 120 μg/ml of 5-methyltryptophan. At 15 min, P^{32}-orthophosphate (1 mc/mg P) was added. At 50 min the culture was mixed with an equal volume of a warmed solution containing 0.2 *M* NaCl, 0.2 *M* phosphate of pH 7, 0.1 *M* KCN, and 0.6% sodium dodecylsulfate. The cells lysed within 30 min at 37°C; unlabeled phage particles were added if desired, and the mixture was extracted with an equal volume of water-saturated phenol by rotation for 30–60 min at 6°C. After centrifugation, and withdrawal of the phenol phase, the extraction was repeated, this time removing any insoluble material formed at the interface. The aqueous phase was sometimes freed of phenol by washing on the rotator with ether, and the ether removed by blowing air over the surface of the solution. After the extract was made 0.6 *M* in NaCl, it was passed into a fractionating column,[4] and low-molecular-weight materials were washed through with 0.6 *M* NaCl. DNA was recovered from the column by elution under a concentration gradient of NaCl.

Extraction by rotation, referred to above, was carried out by attaching tubes or bottles at an angle of about 10° from the horizontal (sufficient to prevent oscillation of their contents) to a wheel that turned them about their long axes at 60 rpm. At all other stages of extraction and chromatography, precautions were taken to avoid unnecessary shear.

Tritium-labeled phage particles were prepared by adding H^3-uridine (0.03 μM/ml, 3 mc/μM) to a bacterial culture[1] infected 7 min earlier. DNA extracted from the purified phage particles showed about 5×10^4 disintegrations/μg-min.

Zone centrifugation was observed in density gradients of sucrose.[5] About 2.5 μg of labeled DNA in 0.1 ml of 0.1 *M* or 0.7 *M* NaCl was placed on 5 ml of sucrose dissolved in the same solvent as the DNA. After the tubes were spun for 30×10^8 rpm^2-hr, their contents were sampled by drop collection and radioactivity was assayed in a scintillation spectrometer.[6]

In making density measurements, solid CsCl was added to solutions of DNA to yield a density of 1.65 at 25°C. Samples of 4.0 ml were spun at 10°C in the SW39 rotor of a Spinco model L centrifuge. The contents of the tubes were sampled and assayed as described above.

For analysis of nucleotide composition, a mixture of P^{32}-labeled DNA and unlabeled reference DNA was hydrolyzed with *Escherichia coli* phosphodiesterase[7] (exonuclease). Nucleotides were separated on a Dowex 1-X8 resin column (1 cm × 60 cm) under gradient elution with 0.01 *M* to 1 *M* ammonium acetate at pH 4.5. More than 90% of the radiophosphorus in the material subjected to hydrolysis was recovered as mononucleotides.

Denatured DNA refers, in this paper, to DNA heated for 10 min at 100°C in 0.1 *M* NaCl at 10 μg/ml and cooled in ice water.

DNA solutions were stirred for 30 min at a concentration of 25 or 30 μg/ml (mainly carrier DNA isolated from phage particles) in a high-speed stirrer.[8]

When not otherwise specified, salt solutions were buffered at pH 6.7 with 0.05 *M* phosphate.

Results.—Labeled DNA isolated as described may be called replicative DNA to distinguish it from the static form found in phage particles. DNA extracted from phage particles, consisting of linear structures of molecular weight 130 million,[9,10] serves as the reference to which replicative DNA is compared below.

Yield and chromatographic properties: About 50 per cent of the total labeled DNA in the infected bacterial cultures is recovered in the phenol extract, and only half of this passes through the fractionating columns. We do not attempt in this paper to account for the losses. The 25 per cent that is recovered elutes from the columns as a rather broad band (0.71–0.73 *M* NaCl), emerging after admixed reference DNA has passed through. The material is heterogeneous, as shown by rechromatography of individual fractions. The chromatographic behavior of replicative DNA can be attributed to an exceptionally high molecular weight or to other peculiarities of structure or composition. However, single-stranded DNA from phage φX174, like ribosomal RNA, elutes at a higher salt concentration than replicative DNA.[11]

RNA content: After initial chromatographic isolation, replicative DNA contains

a variable amount of RNA (up to 50 per cent, measured as alkali-labile radiophosphorus compounds). Chromatographic repurification reduces the RNA content to 10 per cent or less. Neither the chromatographic purification, treatment with pancreatic ribonuclease, nor heating for 30 min at 75°C affects the chromatographic properties of the DNA. Passage of replicative DNA through a fractionating column after previous breakage by stirring, which causes it to elute at a lower salt concentration, readily separates it from the RNA. These facts suggest that much or all of the RNA found is simply a contaminant.

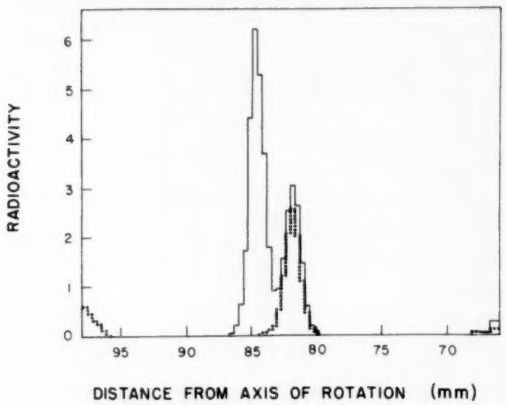

FIG. 1.—Buoyant density in CsCl solution. Solid line, 10 μg of H^3-labeled, heat-denatured reference DNA (left band) and 3 μg of H^3-labeled unheated reference DNA. Dotted line, 1 μg of P^{32}-labeled replicative DNA. Centrifugation for 80 hr at 30,000 rpm.

Density: Replicative DNA and reference DNA band at the same position in a density gradient of CsCl (Fig. 1). Denatured DNA present in the same mixture separates from the native DNA's by a distance of 0.021 gm/cm^3 on the density scale, as expected. One could imagine that the native DNA's, consisting of exceptionally long molecules, would fail to separate from each other in spite of a small density difference. However, a mixture of replicative and reference DNA's, reduced to small fragments by stirring, likewise fails to separate in CsCl. The two DNA's therefore differ by less than 0.001 gm/cm^3 in density, and both must be double-stranded in structure.

Nucleotide composition: Replicative DNA has a nucleotide composition identical to that of reference DNA (Table 1). This result, like the characteristic density, shows that the DNA is phage-specific in origin.

Susceptibility to nucleases: Both replicative DNA and reference DNA are refractory to the action of an exonuclease specific for denatured DNA, and are made sensitive by thermal denaturation (Table 2). Enzymatic tests reveal small differences, however. (1) After thermal denaturation, 85 per cent of the replicative DNA, but only 70 per cent of the reference DNA, is hydrolyzed by exonuclease. This difference disappears when the reference DNA is broken by stirring before de-

TABLE 1

NUCLEOTIDE COMPOSITION (mole %)

	Reference DNA	Replicative DNA
Deoxyadenylate	34	36
Deoxyguanylate	16	14
Thymidylate	32	32
Hydroxymethylcytidylate	19	17
Nonglucosylated	5.6	5.3
Monoglucosylated	12.8	11.1
Diglucosylated	0.6	0.7

TABLE 2

HYDROLYSIS (%) BY ENZYMES SPECIFIC FOR DENATURED DNA

Enzyme	State of substrate	Reference DNA	Replicative DNA
Exonuclease	Native	0–0.1	0–0.1
	Broken	0	1.0–4.0
	Denatured	65–77	81–89
	Broken, denatured	77–88	77–89
Endonuclease and exonuclease	Native	0–0.1	0.4–0.5
	Broken	0	1.1–1.2
	Denatured	87–93	94–99

Differently labeled DNA's of the two kinds were subjected to the treatments indicated, digested, and analyzed, in mixture. The ranges shown include results of several experiments, and results after digestion for different times (40–80 min) in each experiment.

naturation. (2) A mixture of endo- and exonucleases, both specific for denatured DNA, releases about 0.5 per cent of the nucleotides of replicative DNA but not of reference DNA. (3) The difference last mentioned can also be seen with exonuclease alone, after both DNA's have been broken by stirring.

These results support the conclusion that replicative DNA is mainly double helical in structure. However, there are small regions in replicative DNA, but not in reference DNA, that become sensitive to the action of an exonuclease after treatment with agents that break phosphodiester bonds. Probably replicative DNA contains short segments in which the bases are not paired.

Sedimentation rate: Replicative DNA sediments faster than reference DNA in a density gradient of sucrose. As examples, a chromatographic fraction that eluted at 0.71 *M* NaCl sedimented 1.21 times faster than H^3-labeled reference DNA present in the same tube, and a fraction that eluted at 0.73 *M* sedimented 1.35 times faster than the reference. First of all, these results confirm the heterogeneity of replicative DNA, and second, they show that among components of replicative DNA sedimentation rate and chromatographic behavior are correlated in the manner expected for linear DNA molecules of different length. If replicative DNA consists of linear structures, their molecular weights in the examples cited are 1.7 and 2.3 times that of reference DNA.[12] For more compact structures, the molecular weights would be less.

Breakage under shear: When a solution of reference DNA is stirred at a series of increasing speeds, and samples are analyzed chromatographically after each stirring, no change is observed until a critical speed is reached.[8] This speed proves in general to be an inverse measure of the lengths of DNA molecules.[13] On continued stirring at the critical speed, reference DNA breaks progressively into half-length fragments. After partial breakage, therefore, one sees two individually characteristic chromatographic bands. This behavior characterizes a DNA that is initially homogeneous in molecular weight and fragility.

The chromatographic behavior of replicative DNA differs from that of reference DNA in two respects (Fig. 2). First, its chromatographic properties are altered after stirring at 650 rpm, whereas a speed of 1,500 rpm is required to break reference DNA present in the mixture described in the figure. Second, stirring at the critical speed produces not one but two new chromatographic bands. This complicated behavior does not seem to be related to the initial heterogeneity of the material, since the results are similar when the experiment is performed with the total replicative DNA or with narrow chromatographic fractions.

Replicative DNA clearly consists of extremely fragile structures, which tend to yield chromatographically characteristic species on breakage. The fragility supports other evidence that the material is of high molecular weight. The significance of the characteristic breakage products is clear only in a negative sense: they do not resemble molecules of reference DNA, nor do they resemble half-length fragments derived from such molecules.

Sedimentation analysis of the products of breakage yields results generally consistent with those observed by chromatography (Fig. 3). The sedimentation rate begins to decrease after stirring at 500 rpm. As the speed of stirring is increased, the sedimentation rate falls progressively, with no sign of the characteristic fragments recognizable chromatographically. After stirring at 1,800 rpm, at which speed the reference DNA begins to break, the replicative DNA present in the mixture sediments as a broad band centered between the positions of unbroken molecules and half-length fragments of reference DNA, more or less as one would expect as the result of breakage of initially very long, nonuniform structures. The failure to find distinctively sedimenting species among the breakage products again suggests that replicative DNA is not made up of characteristic subunits resembling reference DNA, and perhaps signifies that the chromatographic species illustrated in Figure 2 are characterized by some property other than molecular weight.

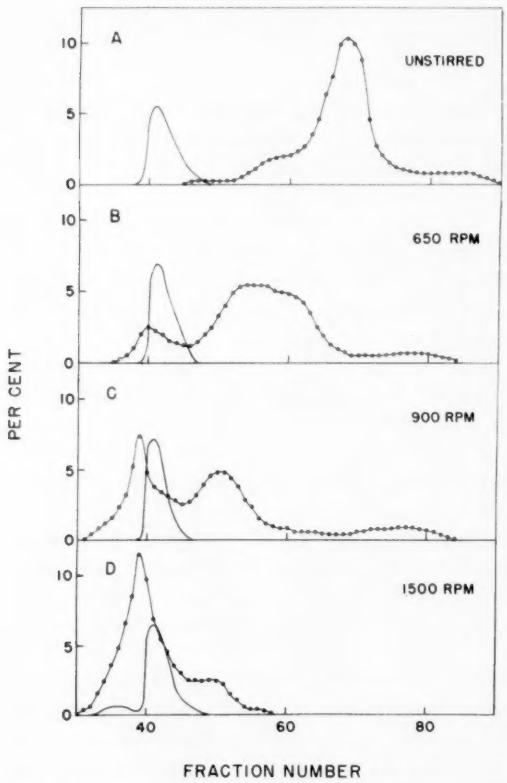

FIG. 2.—Effects of stirring on chromatographic properties. Solid line, unlabeled reference DNA (scaled down for clarity). Dotted line, P^{32}-labeled replicative DNA, representing the 30 per cent eluted last during the chromatographic purification. The two DNA's were stirred in mixture. The reference DNA is just beginning to break at the highest stirring speed.

Equivalent fragments of replicative DNA and reference DNA: Replicative DNA has proved to differ in structure from reference DNA in three respects: it elutes from a column at a higher salt concentration, is altered at lower rates of shear, and sediments faster. These differences are understandable if replicative DNA consists of unusually long, conventional DNA molecules, but they do not rule out more complex structures. The difficulty is that no reference DNA of sufficient length is

available for comparison. This difficulty disappears after the replicative DNA itself has been fragmented, a consideration that suggested experiments of the following type.

Figure 4 shows the chromatographic analysis of a mixture of differently labeled replicative and reference DNA's stirred at 1,800 rpm, the same sample whose sedimentation pattern is seen in Figure 3*D*. Both types of analysis show that only a small amount of the reference DNA was broken, whereas all the replicative DNA was reduced to a collection of fragments whose apparent lengths span an approximately threefold range downward from that of reference DNA molecules. Fractions 42 and 48 of Figure 4 were chosen for further study, with the following results.

Fraction 42 contained fragments of reference DNA somewhat longer than half the original molecular length, together with fragments of replicative DNA with matching chromatographic properties. When a sample of the mixture was subjected to zone sedimentation, the two components sedimented together. One can infer that the fragments of different origin are generally similar in structure.

Fraction 48 contained natural molecules of reference DNA, and fragments of replicative DNA with matching chromatographic properties. The two components of the mixture sedimented together, except for a small, not unexpected, contamination with smaller fragments derived from the replicative DNA. When the mixture was stirred and again subjected to zone sedimentation, 25 per cent of the reference DNA proved to have been broken, and a similar fraction of the replicative DNA had been altered so that it sedimented with the broken reference DNA. Thus, among fragments produced by stirring replicative DNA, one can find some that resemble reference DNA in chromatographic properties, sedimentation rate, and fragility under shear, indicating very similar structures for the two materials.

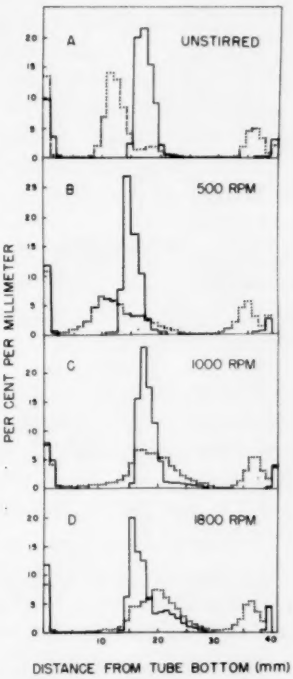

FIG. 3.—Effects of stirring on sedimentation rate. Solid line, H^3-labeled reference DNA. Dotted line, P^{32}-labeled replicative DNA, representing the central 30 per cent eluted during chromatographic purification. The two DNA's were stirred in mixture and sedimented through concentration gradients of sucrose.

We infer that, whatever the original structure of replicative DNA may be, it yields under shear fragments of typical linear structure, of normal fragility per unit length, and predominantly of lengths that could arise only by breakage of structures longer than molecules of reference DNA.

Discussion.—At present we can recover only a small fraction of the DNA content of infected bacteria in a form subject to study by our methods. The recovered DNA is heterogeneous but consists exclusively of structures apparently exceeding 130 million in molecular weight. Thus the fragmentation of polynucleotides during

phage growth[14] occurs without release of the ultimate fragments or, possibly, is suppressed by the inhibitors of protein synthesis used in our experiments.

The finding of large DNA structures in infected bacteria raises several questions. Are they the typical precursors of the DNA in phage particles, or have other structures that are the precursors been missed? Does their large size reflect a transient replicating state in which they were caught at the moment of extraction, or are they essentially finished structures of redundant length, from which segments must be cut before insertion into phage particles? What are the lengths of the polynucleotide chains of which the large structures are composed? All these questions call for new or refined analytical techniques.

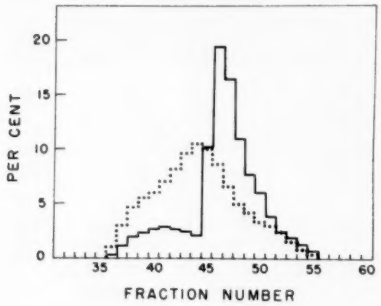

FIG. 4.—Chromatographic separation of DNA fragments. Solid line, H^3-labeled reference DNA. Dotted line, P^{32}-labeled replicative DNA. The mixture had been stirred at 1,800 rpm. Its sedimentation is shown in Figure 3*D*.

Summary.—DNA extracted from T2-infected bacteria in which the terminal stages of phage growth are prevented by an inhibitor of protein synthesis shows three properties indicative of unusually high molecular weight: strong binding to a column of basic protein, rapid sedimentation, and extreme fragility under hydrodynamic shear. With respect to nucleotide composition, fragility per unit length, and susceptibility to thermal denaturation, it resembles the DNA found in phage particles, but differs again from that DNA in showing signs of physical inhomogeneity and incomplete double-helical structure.

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*ISOTOPIC STUDIES ON ESTROGEN-INDUCED ACCELERATIONS OF RIBONUCLEIC ACID AND PROTEIN SYNTHESIS**

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When the immature or mature estrogen-deficient rat is given a dose of estrogenic steroid, a constellation of biochemical and physiological reactions are initiated in the uterus. Among uterine responses to estrogen are the well-known initiations of fluid imbibition[1, 2] and the accelerations of synthesis of phospholipids, nucleic acids, and proteins.[3] This system, representing a relatively rapid interaction between mammalian cells and a hormonal stimulus, is a valuable one for experimental attack on the general problem of discovery of the biochemical steps through which physiologic concentrations[4, 5] of hormones exert their characteristic influences on tissues having competence for extensive as well as specific responses to such.

Several kinds of evidence now indicate that the previously sought concept of "a" mechanism for steroid hormone action is an interpretation more apparent than real.[6, 7] Existing theories on mechanisms of steroid hormone action can, for the most part, be divided into two categories: those concerned with reactions involved in ways still uncertain with extranuclear components such as dehydrogenases, transhydrogenases, oxidases, peroxidases, or histamines,[8–14] and those concerned with initiations or accelerations of synthetic processes.[15–17] The latter have been advocated and tested in particular by G. C. Mueller and co-workers. Recently, they have reported[18] *in vivo* studies indicating that combined treatments of individual, ovariectomized rats with puromycin (a known inhibitor of protein synthesis) and 10 micrograms of estradiol-17b lead in 4 hr to these situations for previously atrophic uteri: (*i*) suppression of protein synthesis to levels below control levels as well as below those expected to result from estrogen stimulation alone and (*ii*) retardation of the increases in RNA and phospholipid synthesis which normally follow such hormonal stimulation. Suppressions of RNA and phospholipid synthesis to levels below those of controls were not observed. That these workers administered to experimental animals doses of estradiol far above those expected to be operative at the tissue level (10 μg being equivalent to at least 100 Allen-Doisy units) raises the possibility that their findings reflect a nonphysiological situation or, perhaps, represent uterine responses atypical in comparison with those for lower doses.

Below are reported experiments which show or permit the following: (*a*) a confirmation at a lower level of hormonal stimulation of the general picture for early estrogen action found by Mueller *et al.*;[18] (*b*) a demonstration which, for the range of hormone doses tested, indicates that acceleration of RNA synthesis is intimately associated with the acceleration of protein synthesis which results from estrogenic stimulation; (*c*) a quantification—which complements for comparative studies on three naturally occurring estrogens the previous findings of Hisaw, Jr.,[2]—of some minimum steroid doses necessary for protein synthesis via this system; and (*d*) a discussion of the hypothesis that intranuclear reactions and RNA synthesis are

involved in the inceptive phase of the early action (i.e., the period prior to acceleration of protein synthesis) of estrogen on the uterus.

Materials and Methods.—All experiments employed immature female rats (Wistar-mixed strain) 30–32 days old and weighing 62–65 gm. Ovariectomy of females by the mid-ventral approach was performed 5–6 days prior to experimentation. Steroids (estradiol-17b, estriol, and estrone), with doses dissolved in 0.05 ml of a 1% ethanol-saline solution, were administered intravenously at the beginning (zero hr) of all experiments. Puromycin, 5 mg in 0.5 ml buffered saline, was injected intraperitoneally at zero, 1, 2, and 3 hr in all experiments utilizing this inhibitor. Following injections at zero hr of isotopes as well as of steroids and/or puromycin, animals were allowed a 4-hr period[18] for uterine responses, killed by ether exposure, and the uteri dissected out. Uteri were then stripped of fat and connective tissue, blotted on a damp cloth, weighed, and transferred to ice-cold TCA in tubes for glass-to-glass homogenizing by hand. Phosphate-P^{32}, 500 microcuries in 0.05 ml buffered saline, was injected intraperitoneally only at zero hr of experiments, and glycine-2-C^{14}, 4–5 microcuries (3.06 millicuries per millimole) in 0.5 ml buffered saline, was injected intraperitoneally at zero, 1, 2, and 3 hr.

For determination of glycine-2-C^{14} incorporation into uterine protein, fractions were extracted via standard 5% TCA washings, followed by extraction of the lipid fraction by suspension in 80% ethanol, 100% ethanol, chloroform-ethanol (2:1 v/v), and ethyl ether (twice) as described by Aizawa and Mueller.[19] Sample protein fractions were then transferred via Hooker-Forbes microsyringe delivery to aluminum planchettes. These were dryed at 90°C and counted in a continuous gas flow counter. Corrections for background counts (self-absorption not corrected for) were made, and activity expressed as counts per minute (cpm) per milligram of extracted uterine protein.

Estimates of P^{32} incorporation into uterine RNA are based on alkaline digestion of protein-nucleic acid fractions following, for comparative purposes, the technique described by Mueller *et al.*[18] This was modified only to the extent that following elutions of nucleotide (AMP, CMP) spots from chromatographs, samples of such were transferred to planchettes by Hooker-Forbes

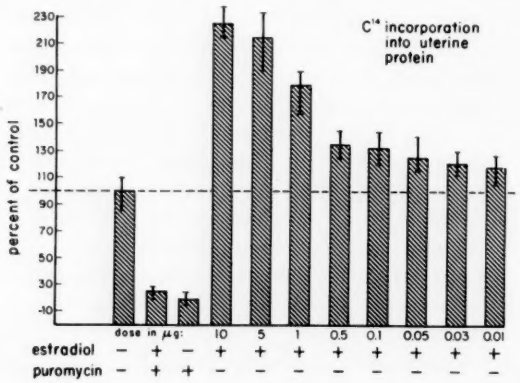

FIG. 1.—Uterine response to graded doses of estradiol-17b and incorporation of glycine-2-C^{14} into uterine protein. Brackets denote ranges, and each bar indicates a mean value, expressed as per cent of control (left bar which is 311 cpm/mg protein) for extracted, C^{14}-incorporated protein from 10 uteri. Five rats were used for each of the experiments described here and in Figures 2–6. All puromycin doses were the same: 5 mg injected intraperitoneally in buffered saline at zero, 1, 2, and 3 hr (see *Methods and Materials*). For Figures 1–6, doses of steroid are listed for each bar immediately beneath the horizontal axis of each diagram. Also, for experiments involving + puromycin and + estrogen, the dose of steroid is the same (10 μg) for each estrogen tested.

microsyringe delivery. Samples were counted by a closed end window geiger counter, with activity (following corrections) being recorded in cpm per micromole of 2′, 3′ AMP.

Results.—(a) *Inhibition of protein synthesis and its influence on uterine protein synthesis in control and estrogen-stimulated animals:* No attempt was made to determine the dose-response relation for graded doses of puromycin on the estrogen-stimulated uterus. Instead, in preliminary experiments, a large, arbitrarily-selected dose of puromycin (5 mg), injected at zero, 1, 2, and 3 hr, was found (Fig. 1) to suppress uterine protein synthesis to levels significantly below those for estradiol-treated animals as well as for controls. This procedure was used in all experiments here reported, and the observations cited were subsequently found to be true for experiments where estriol or estrone was coadministered with puromycin (Figs. 3, 5). It will be noted that the results reported (Figs. 1, 3, 5) indicate for the three steroids coadministered with puromycin that, in each case, the suppressed rate of C^{14} incorporation into uterine protein is somewhat greater than for puromycin-treated controls—thus indicating that even these massive doses of puromycin do not inhibit completely the ability of these estrogens to induce protein synthesis.

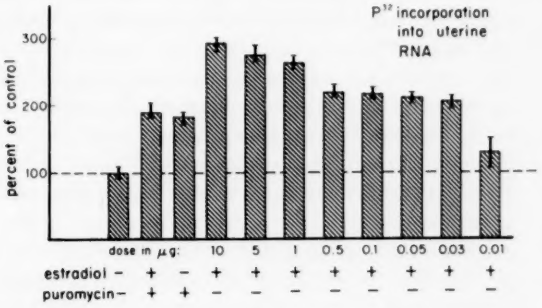

FIG. 2.—Uterine response to graded doses of estradiol-17b and incorporation of P^{32} into uterine RNA. Bar values reported as per cent of control value (1442 cpm/micromole 2′, 3′ AMP; see *Materials and Methods*).

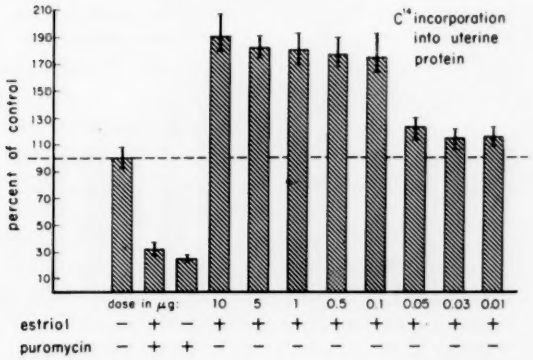

FIG. 3.—Uterine response to graded doses of estriol and incorporation of glycine-2-C^{14} into uterine protein. Bar values reported as per cent of control value (311 cpm/mg protein).

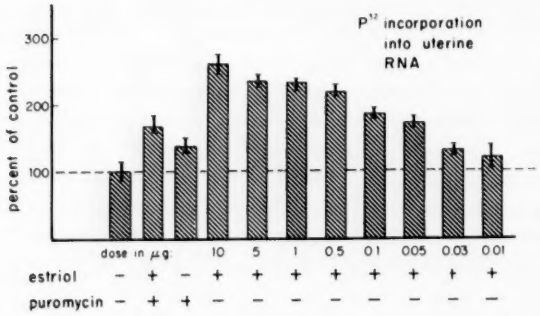

FIG. 4.—Uterine response to graded doses of estriol and incorporation of P^{32} into uterine RNA. Bar values reported as per cent of control value (1442 cpm/micromole 2′, 3′ AMP).

(b) *Influence of inhibition of protein synthesis on the ability of the uterus to accelerate RNA synthesis following estrogen stimulation:* Simply stated, in all experiments where puromycin was administered to animals in conjunction with estrogen treatments, incorporation of P^{32} into uterine RNA was accelerated. Furthermore, such RNA labeling was also accelerated in puromycin-treated controls (Figs. 2, 4, 6). The latter observation, although observed previously[18] and still unexplained, is of particular interest in the light of recent work on the mechanism of protein synthesis in microorganisms, and, along with information cited below, hints at the possibility of RNA synthesis being a key step in the early phase of estrogen action in the uterus (see *Discussion*).

(c) *Comparative effectiveness of estradiol-17b, estriol, and estrone on uterine protein and RNA synthesis:* The observation that certain estrogens initiate a rapid growth response in the rat uterus is, of course, a classic one in physiology, and by it is implied that estrogens in some way lead to increased rates of protein synthesis. A question that still remains unexplained is: through what biochemical steps, and

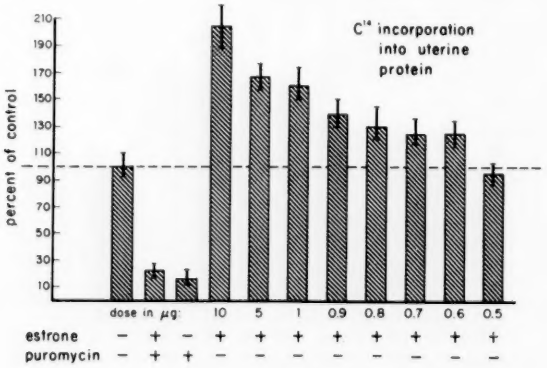

FIG. 5.—Uterine response to graded doses of estrone and incorporation of glycine-2-C^{14} into uterine protein. Bar values reported as per cent of control value (311 cpm/mg protein).

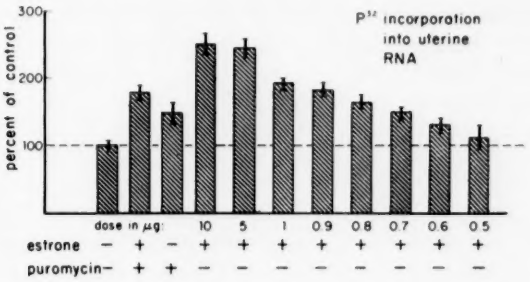

FIG. 6.—Uterine response to graded doses of estrone and incorporation of P^{32} into uterine RNA. Bar values reported as per cent of control value (1442 cpm/micromole 2′, 3′ AMP).

how soon following the arrival of estrogenic molecules at the site of the estrogen-deficient uterus, such steroids lead to accelerations of protein synthesis? On these grounds, and because Mueller *et al.*[18] used such a large dose (10 μg) of estradiol in their experiments, an effort was made to determine the minimum dose of estrogen necessary for acceleration of uterine protein and RNA synthesis in this system. That Hisaw, Jr.,[2] had estimated minimum steroid doses necessary for the Astwood uterine response in the immature rat was an additional stimulus for making these quantifications.

Although there is some overlap for the range of uterine responses for different doses (for example, see Fig. 1) of the same steroid, the minimum doses necessary for apparent increase over control rates of C^{14} incorporation into uterine protein are as follows: estradiol, 0.03 μg; estriol, 0.05 μg; estrone, 0.6 μg. For increases over control rates of incorporation of P^{32} into uterine RNA, the following values are reported: estradiol, 0.03 μg; estriol, 0.05 μg; estrone, 0.6 μg. Thus, working with doses of steroids near minimum for *in vivo* estrogenic stimulation of uteri via intravenous injection, no evidence is found for estrogenic acceleration of uterine protein synthesis without concomitant acceleration of uterine RNA synthesis.

The problem of how much of the injected steroid actually becomes operational at the site of the rat uterus (see Jensen and Jacobson[4]) cannot be evaluated on the basis of these experiments. It is of interest, however, that the values for minimum doses of estrogen necessary for acceleration of uterine synthetic processes are of a size order comparable to those found by Hisaw, Jr.[2] And, although the data here reported differ quantitatively, they seem to support qualitatively the preceding worker's conclusion that estradiol and, to a lesser extent, estriol, are more effective for induction of uterine growth than estrone.

Discussion.—The experiments described in the preceding paragraphs confirm the findings of Mueller *et al.*[18] reported previously. As such, the data here reported demonstrate that acceleration of protein synthesis is an essential feature of early estrogen action in the uterus. In addition, they indicate that the processes involved are elicited by much smaller doses of estrogen than those used by the workers' cited.[18] Puromycin, by suppressing protein synthesis apparently at cytoplasmic ribosomal sites, inhibits the ability of estrogen to exert its characteristic growth-inducing influence on the uterus. This is true if the criterion for such is

either increased protein synthesis or fluid imbibition.[18] On general theoretical grounds, this observation poses two possibilities, each of which is subject to experimental analysis: (*a*) that estrogen in some way alters the cytoplasmic biochemical environment so as to facilitate or favor protein synthesis by ribosomes; or (*b*) that estrogen directly or indirectly participates in intranuclear reactions—the results of which (synthesis of messenger and/or [?] soluble RNA) thus regulate protein synthesis.

Four lines of evidence may be cited to support hypothesis (*b*): (*i*) As shown by Mueller *et al.*,[18] and confirmed in the present paper, inhibition of protein synthesis by puromycin treatment of controls or of estrogen-treated animals leads to acceleration of uterine RNA synthesis (as estimated by P^{32} incorporation) to levels over those of controls. Since puromycin blocks protein synthesis by interference with cytoplasmic ribosomes, and since protein synthesis in mammalian cells is now considered to be regulated by RNA passing from the nucleus to the cytoplasm, then blockage of protein synthesis with puromycin might not interfere with RNA synthesis (soluble as well as messenger). Thus, increased P^{32} incorporation into uterine RNA four hr following puromycin treatment might reflect a pre-block accumulation of moderately stable as well as unstable RNA. However, there presently exists no information on the degree of lability of uterine RNA, and an alternate possibility here would be that puromycin results in increased labeling of RNA by interference with some control mechanism for the synthesis of messenger RNA. The need for studies on the characterization of the kinds of uterine RNA induced by estrogenic stimulation is obvious. (*ii*) As here reported for *in vivo* experiments using intravenous injections and minimum doses of estrogen necessary for acceleration over controls of uterine synthetic processes, no evidence is found for acceleration of protein synthesis without concomitant acceleration of RNA synthesis. (*iii*) Further experiments[20] in the writer's laboratory—studying estrogen-induced accelerations of uterine RNA and protein synthesis as respectively correlated with time from zero hour—show that RNA synthesis is accelerated prior to protein synthesis. (*iv*) Very recent findings, reported by Liao and Williams-Ashman,[21] demonstrate that the androgen environment in the male rat is apparently associated positively with the production, if not operation, of prostatic messenger RNA.

The findings and interpretations presented above suggest that it would be fruitful to test the theory that estrogen initially operates on the uterus by intranuclear reactions with resulting production or regulation of messenger RNA. For this, a variety of experimental approaches are now available, and studies of cell-free systems, *in vivo* time-differentials for uterine responses, and actinomycin D sensitivity are logical lines of investigation.

Summary.—The 1961 *in vivo* experiments and findings of Mueller and co-workers have been repeated and confirmed for a lower range of hormonal stimulation. Puromycin inhibits uterine protein synthesis, but not uterine RNA synthesis, to levels below those of non- or estrogen-treated controls. Working with near minimum doses for three naturally occurring estrogens, and thus complementing the previous findings of Hisaw, Jr., no evidence is found for estrogen-induced acceleration of protein synthesis without concomitant acceleration of RNA synthesis. Evidence is cited for the hypothesis that intranuclear reactions and RNA

synthesis are inceptive events of early estrogen action which precede the acceleration of protein synthesis resulting from such hormone action.

* I am particularly grateful to F. L. Hisaw and H. S. Forrest for useful suggestions and assistance in various ways. I am also much indebted to R. Williamson and Mrs. B. Moore for extensive technical assistance.

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The following abbreviations are used: DNA for desoxyribonucleic acid; RNA for ribonucleic acid; TCA for trichloroacetic acid; AMP for adenosine monophosphate; CMP for cytodine monophosphate

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THE BIOCHEMICAL FUNCTION OF BIOTIN, VI. CHEMICAL STRUCTURE OF THE CARBOXYLATED ACTIVE SITE OF PROPIONYL CARBOXYLASE†*

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In 1959 Lynen *et al.*[1] reported that β-methylcrotonyl carboxylase catalyzes the carboxylation of free (+)-biotin when it is substituted for the natural substrate, β-methylcrotonyl CoA.[2] The carboxylation product was isolated[3] as its dimethylester and identified as 1′-N-carboxy-(+)-biotin. Their proposed mechanism visualized enzyme-bound biotin in the form of an allophanic acid derivative, 1′-N-carboxy-(+)-biotinyl-enzyme, as the active "carboxyl-transfer" intermediate in

this and other related carboxylation reactions. This view has been strongly supported[4] by β-methylcrotonyl carboxylase-catalyzed exchange reaction studies with various labeled substrates. Recently Knappe *et al.*[5] degraded $C^{14}O_2$-enzyme (β-methylcrotonyl carboxylase) by brief treatment with trypsin, followed by methylation with CH_2N_2, and then hydrolysis with biotinidase. Essentially all of the C^{14}-activity originally present in $C^{14}O_2$-enzyme was recovered as 1′-N-carbomethoxy-(+)-biotin thus confirming the original hypothesis of Lynen *et al.*[1]

Propionyl carboxylase from pig heart and bovine liver mitochondria have been extensively investigated and found[6-9] to possess enzymatic properties similar to those of β-methylcrotonyl carboxylase. The exchange reactions catalyzed,[6, 8, 9] the stoichiometry between biotin content and $HC^{14}O_3^-$ bound to form $C^{14}O_2$-enzyme,[7] and the stability properties of $C^{14}O_2$-enzyme[7] all evidence this similarity. It is likely therefore that the nature of the HCO_3^- binding sites of the two enzyme types are the same.

The purpose of the present investigation was to determine the chemical structure of the carboxylated active site of propionyl carboxylase. Evidence presented in this report has led to the identification of this site as ε-N-(1′-N-carboxy-(+)-biotinyl)-lysyl-enzyme.

Materials and Methods.—Propionyl carboxylase from bovine liver mitochondria was purified through the second ammonium sulfate fractionation (Step 5) according to the method of Lane and Halenz.[10] The specific activity of the carboxylase was 3.2 units per mg of protein. One unit of carboxylase catalyzes the carboxylation of 1.0 μmole of propionyl CoA per min at pH 8.0 and 25° using the spectrophotometric assay.[11]

Biotin was determined by the yeast growth method[12] (*Saccharomyces cerevisiae strain* 139) after hydrolysis of the enzyme preparations with 3.6 *N* H_2SO_4 for 1 hr at 120° in an autoclave.

Biotinidase was assayed and purified from pig kidney according to methods provided by Dr. J. Knappe (Univ. of Heidelberg). The biotinidase preparation (100-fold purified) used in these experiments had a specific activity of 6.2 units per mg of protein. One unit of biotinidase catalyzes the hydrolysis of 1.0 mμmole of N-(+)-biotinyl-p-aminobenzoate per min at pH 6.0 (phosphate buffer) and 37°.

Pronase, a proteolytic enzyme isolated from *Streptomyces griseus*, was obtained from the California Biochemical Corp.

The 1′-N- and 3′-N-carbomethoxy-(+)-biotin methyl esters were prepared by the method of Knappe *et al.*[3] Biocytin and 1′-N-carbomethoxybiocytin were generously provided by Dr. J. Knappe (Univ. of Heidelberg).

Results.—Preparation of $C^{14}O_2$-enzyme and carboxyl transfer to propionyl CoA: $C^{14}O_2$-enzyme was formed by short-term incubation of propionyl carboxylase with $HC^{14}O_3^-$, ATP, and Mg^{++} as described in the legend for Table 1. After addition of EDTA to prevent enzymatic exchange of unlabeled HCO_3^- with $C^{14}O_2$-enzyme in subsequent steps, the reaction mixture was applied to a Sephadex G-50 column and the eluate fractions containing the $C^{14}O_2$-enzyme, free of all other reaction mixture components, were retained for the experiments to be described. The quantity of carboxyl transferred from $C^{14}O_2$-enzyme to propionyl CoA and of biotin in $C^{14}O_2$-enzyme were compared. Aliquots of the $C^{14}O_2$-enzyme preparations were incubated with propionyl CoA; mμmoles of carboxyl transferred to form methylmalonyl

TABLE 1

$C^{14}O_2$-ENZYME: CARBOXYL TRANSFER TO PROPIONYL CoA, BIOTIN CONTENT, AND METHYLATION

Experiment number	Protein (mg)	C^{14}-carboxyl transfer to propionyl CoA (cpm)	(mμmoles)	Biotin content* (mμmoles)	Methylated $C^{14}O_2$-enzyme recovered in Pronase digest† (cpm)
1	18.9	256,000 (910)‡	22	30	153,000
2	21.2	390,000 (1,100)‡	33	34	173,000

$C^{14}O_2$-enzyme was prepared by incubating propionyl carboxylase (specific activity, 3.2 units per mg; 24.1 and 25.0 mg in Experiments 1 and 2, respectively) with the following components (in μmoles): Tris·HCl, pH 8.4, 50; GSH, 2.5; ATP, 5; $MgCl_2$, 5; and $KHC^{14}O_3$ (11,800,000 cpm per μmole), 2.8 in a total volume of 3.8 ml at 1°–2° for 4 min. The specific activity of $HC^{14}O_3^-$ was accurately determined from the total radioactivity incorporated into methylmalonyl CoA and the quantity of ADP formed when an aliquot (1.0 ml) of the complete reaction mixture described above was incubated with propionyl CoA (1.8 μmoles) for 3 min at 25°. Specific activity of $HC^{14}O_3^-$ = cpm incorporated into methylmalonyl CoA/μmoles of ADP formed. The total C^{14}-activity incorporated into methylmalonyl CoA was determined from the specific activity of methylmalonic acid recrystallized to constant specific activity after addition of carrier and alkaline hydrolysis. ADP was determined using the coupled pyruvate kinase-lactic dehydrogenase system and following DPNH oxidation spectrophotometrically. The $C^{14}O_2$-enzyme-containing reaction mixture was applied to a 2 × 15 cm Sephadex G-50 column and the column was washed with 0.01 *M* Tris·HCl, pH 8.4. Fractions containing $C^{14}O_2$-enzyme, which were completely resolved from the other reaction mixture components, were pooled (total volume, 10 and 8.5 ml in Experiments 1 and 2, respectively). Aliquots of the $C^{14}O_2$-enzyme solution were taken for counting, for carboxyl-transfer to propionyl CoA, and for 4 *N* H_2SO_4 treatment. Aliquots (0.4 ml) for carboxyl transfer to propionyl CoA were incubated (25° for 4 min and 1°–2° for 4 min in Experiments 1 and 2, respectively) with (in μmoles): Tris·HCl, pH 8.4, 50; GSH, 2; EDTA, 10; and propionyl CoA, 0.6 in a final volume of 0.83 ml. Millimicromoles of C^{14}-carboxyl-transfer were determined from the acid-stable radioactivity and specific activity of the $HC^{14}O_3^-$ used for $C^{14}O_2$-enzyme formation.

* Biotin content of the enzyme was 0.387 μg per mg of protein (determined as described in *Materials and Methods* section).

† Methylation was accomplished with CH_2N_2 as described in test.

‡ Figures in parentheses represent nonvolatile C^{14}-activity after subjecting $C^{14}O_2$-enzyme to 4 *N* H_2SO_4 hydrolysis for 30 min at 100°.

CoA were calculated from acid stable radioactivity and the accurately-determined specific activity of the $HC^{14}O_3^-$ employed (see Table 1 for details). Treatment of aliquots of the $C^{14}O_2$-enzyme solution with 1 *N* acetic acid at 25° for several min or 4 *N* H_2SO_4 at 100° for 30 min (see Table 1) resulted in essentially complete loss of radioactivity. The biotin content of the carboxylase preparation used in these experiments was determined as described in *Materials and Methods*. As shown in Table 1 there is good agreement between the quantity of carboxyl transferred from $C^{14}O_2$-enzyme to propionyl CoA and the quantity of $C^{14}O_2$-enzyme-bound biotin. The higher temperature (25° for 4 min) during carboxyl transfer in Experiment 1 compared to Experiment 2 (1–2° for 4 min) appears to be responsible for the somewhat lower carboxyl transfer relative to biotin content in Experiment 1. This can be attributed[7] to an increased rate of decarboxylation of $C^{14}O_2$-enzyme at the higher temperature and the consequent shift in reaction equilibrium leading to the decarboxylation (loss of C^{14}-label) of methylmalonyl CoA. Hence, apparent carboxyl transfer from $C^{14}O_2$-enzyme to propionyl CoA is somewhat lower than expected. Nevertheless, the results of these experiments indicate that 1.0 mole of transferable carboxyl is bound per mole of enzyme-bound biotin and are in agreement with those of Kaziro and Ochoa.[7]

Methylation of $C^{14}O_2$-enzyme, enzymatic hydrolysis of the methylated protein, and identification of the C^{14}-labeled hydrolysis product: Stabilization of the labile carboxyl group of $C^{14}O_2$-enzyme by methylation with CH_2N_2 was necessary to prevent its loss during the subsequent proteolytic digestion of the C^{14}-labeled enzyme complex. Kosow and Lane[13] had previously found that C^{14}-biotin covalently bonded to native propionyl carboxylase could be quantitatively recovered as biocytin (ε-N-(+)-biotinyl-lysine) following digestion of the labeled carboxylase with Pronase (*Streptomyces griseus* protease). It was concluded that biotin is attached to carboxylase through lysyl ε-amino groups of the enzyme. In a similar manner the

methylated $C^{14}O_2$-enzyme was subjected to the proteolytic action of Pronase to determine whether the predicted C^{14}-labeled 1′-N-carbomethoxy-biocytin fragment would be released.

A nine milliliter aliquot of the $C^{14}O_2$-enzyme-containing Sephadex G-50 eluate (231,000 cpm, 17 mg of protein; see legend, Table 1) from Experiment 1 was brought to a final methanol concentration of 85 per cent with ice-cold methanol. The solution was vigorously stirred at 0–2° during the rapid addition of a cold ethereal solution of CH_2N_2 (approximately 30 ml of a 1.0 *M* solution). A persisting yellow color was evident. The enzyme protein, which precipitated during methylation, was recovered by high-speed centrifugation. The sedimented protein was resuspended in a final volume of 7.6 ml containing 200 μmoles of potassium phosphate buffer (pH 7.5), 0.35 ml of ethanol, and 4 mg of Pronase. The proteolytic digestion mixture was incubated for 48 hr at 37°. After several hours of digestion the denatured protein had been completely solubilized, therefore aliquots of the digestion mixture were taken and recovery (60% in Experiment 1) of C^{14}-activity was determined (see Table 1).

The Pronase digest was "streaked" directly on two sheets (90 cm) of Whatman 3 MM paper and chromatographed descending using the *n*-butanol-glacial acetic acid-H_2O (80:20:20, v/v) solvent system. All the radioactivity on the developed chromatograms was located in a single narrow band (R_f = 0.35) which corresponded exactly to 1′-N-carbomethoxybiocytin, but which was not sufficiently resolved from biocytin to warrant its definitive identification. The radioactive band on each chromatogram was cut out and eluted with H_2O. Recovery in the eluate of radioactivity originally present in the Pronase digest was 88 per cent.

Definitive chromatographic identification of the C^{14}-labeled compound was accomplished by use of a second solvent system which clearly resolved biocytin and 1′-N-carbomethoxybiocytin. Aliquots (containing 5000 cpm) of the concentrated eluate were applied to strips of Whatman 3 MM paper and cochromatographed descending with authentic biocytin and 1′-N-carbomethoxybiocytin using n-butanol-H_2O-pyridine-glacial acetic acid (30:24:20:6, v/v) as solvent.[14] The biocytin derivatives were located on the chromatograms with ninhydrin spray reagent and radioactivity with a strip counter. Figure 1 shows the exact correspondence of the single radioactive spot on the chromatogram with authentic 1′-N-Carbomethoxybiocytin (R_f = 0.55) which is definitely resolved from biocytin (R_f = 0.46). This indicates that the C^{14}-labeled fragment released by Pronase action on the methylated $C^{14}O_2$-enzyme is in fact N-carbomethoxybiocytin.

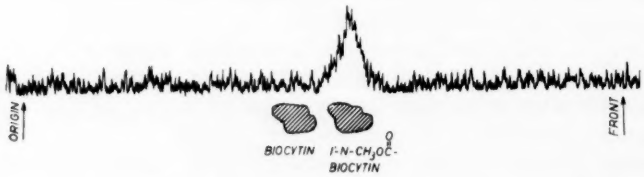


FIG. 1.—Paper chromatographic identification of the C^{14}-labeled compound released by proteolytic digestion of methylated $C^{14}O_2$-enzyme. Shown in the figure are the strip counter plot of radioactivity and locations of cochromatographed authentic compounds (biocytin and 1′-N-carbomethoxybiocytin; R_f values = 0.46 and 0.55, respectively) from the developed chromatogram. See text for details.

Hydrolysis of $C^{14}O_2$-enzyme-derived N-carbomethoxybiocytin by biotinidase and identification of the C^{14}-1′-N-carbomethoxy biotin released: The remaining C^{14}-N-carbomethoxybiocytin-containing eluate, from the initial chromatographic step (n-butanol-glacial acetic acid-H_2O solvent system) described in the preceding section, was subjected to two successive chromatographic purifications prior to incubation with biotinidase. These included: descending chromatography on Whatman 3 MM paper using the n-butanol-H_2O-pyridine-glacial acetic acid solvent system and descending chromatography on water-washed S. and S. No. 2043 *b* paper using the n-butanol-glacial acetic acid-H_2O solvent system. In each case the substances were applied to the paper by "streaking," the chromatogram was developed, and the single radioactive band was eluted. Recoveries in the eluate of applied radioactivity were 80 and 90 per cent, respectively, for the two chromatographic systems.

A portion (20,400 cpm) of the chromatographically-purified C^{14}-labeled N-carbomethoxybiocytin was subjected to enzymatic hydrolysis by biotinidase. The biocytin derivative was incubated for 14 hr at 37° in a final volume of 4.8 ml with 135 μmoles of acetate buffer (pH 6.0), 20 mμmoles of "carrier" 1′-N-carbomethoxybiocytin, 6.0 μmoles of EDTA, and 52 units of purified biotinidase (specific activity, 6.2 units per mg of protein). Additional biotinidase was added during incubation: 26 units at 3 and 6 hr and 30 units at 9 hr. Since biotinidase has a much lower affinity for 1′-N-carbomethoxybiocytin than for the assay substrate, higher enzyme concentrations and addition of carrier 1′-N-carbomethoxybiocytin were necessary for nearly quantitative hydrolysis of the C^{14}-labeled compound. Following incubation, the reaction mixture was applied to a 0.7 × 6.0 cm column of Dowex 50 (×2,200–400 mesh) in the H^+ form after which the column was washed with H_2O. The C^{14}-carbomethoxybiotin liberated during hydrolysis was recovered in 80 per cent yield (19,200 cpm) in the water eluate and the unhydrolyzed C^{14}-N-carbomethoxybiocytin was retained by the resin. After concentration of the water eluate to dryness, the residue was taken up in a small volume of ether, and 5 ml of a 1 *M* ethereal solution of CH_2N_2 was added to methylate the C^{14}-N-carbomethoxy biotin. After concentration to dryness, the methylated product was redissolved in ether saturated with ethylene glycol at 4°. Aliquots containing 3–4,000 cpm were applied to ethylene glycol-impregnated S. and S. No. 2043 *b* paper strips.[3] After application of authentic biotin methylester and 1′-N- and 3′-N-carbomethoxy-(+)-biotin methyl esters, also dissolved in ethylene glycol-saturated ether, the chromatograms were developed for 5 hr at 4° using ether saturated with ethylene glycol as solvent. The authentic compounds were located on the chromatograms with 0.2 per cent $KMnO_4$ spray and radioactivity with a strip counter. As shown in Figure 2 the methylated radioactive fragment formed by biotinidase action on N-carbomethoxybiocytin is chromatographically identical to 1′-N-carbomethoxy-(+)-biotin methylester. Neither biotin methylester nor 3′-N-carbomethoxy-(+)-biotin methylester were labeled. It is apparent therefore that the parent compound hydrolyzed by biotinidase was 1′-N-carbomethoxybiocytin. Since the 1′-N- and 3′-N-isomers were completely resolved and the radioactivity corresponded to the 1′-N-isomer, it can be concluded that carboxylation of propionyl carboxylase occurs at the 1′-N-positions of its biotinyl residues.

Discussion.—The present investigation confirms the earlier finding of Kosow and

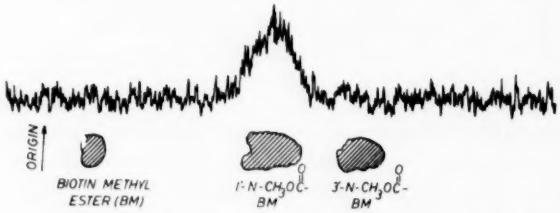
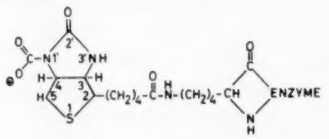


FIG. 2.—Paper chromatographic identification of the C^{14}-labeled product of biotinidase action on $C^{14}O_2$-enzyme-derived N-carbomethoxybiocytin. Shown in the figure are the strip counter plot of radioactivity and locations of cochromatographed authentic compounds (biotin methyl ester and 1'-N- and 3'-N-carbomethoxy-(+)-biotin methyl esters; 3.2, 14.5, and 20 cm from the origin, respectively) from the developed chromatogram. See text for details.

Lane[13] that biotin is bonded to propionyl carboxylase through lysyl ϵ-amino groups of the enzyme. The experiments described show definitively that the active carboxyls of "carboxylated" propionyl carboxylase are attached to the 1'-N-positions of ϵ-N-(+)-biotinyl-lysyl moieties as shown below.



Recently Wakil and Waite[15] proposed that either the ureido carbon atom (2'-position of biotin) of biotinyl-enzyme or the N-carboxy group of enzyme-bound N-carboxy-2-(δ-carboxybutyl)-3,4-diaminothiophane (the diamino carboxylic acid formed by degradation of the urea ring of biotin) serve as the active carboxyl of "carboxylated" acetyl carboxylase. The present investigation with propionyl carboxylase and recent investigations with methylmalonyl-oxalacetic transcarboxylase[16] and β-methylcrotonyl carboxylase[5, 14] are incompatible with this hypothesis. Were the hypothesis applicable to propionyl carboxylase, biocytin, and not N-carbomethoxybiocytin, would have been the C^{14}-labeled fragment obtained when the methylated $C^{14}O_2$-enzyme (propionyl carboxylase) was subjected to proteolytic digestion. As shown in the present investigation N-carbomethoxybiocytin was the only C^{14}-labeled proteolytic digestion product. In addition, degradation of this compound (identified as N-carbomethoxybiocytin) by biotinidase liberated C^{14}-1'-N-carbomethoxy-(+)-biotin and not C^{14}-biotin. When $C^{14}O_2$-enzyme (propionyl carboxylase) was subjected to 4 N H_2SO_4 hydrolysis for 30 min at 100°, essentially no nonvolatile C^{14}-activity remained. Wakil and Waite,[15] on the other hand, found that when $C^{14}O_2$-enzyme (acetyl carboxylase) was subjected to even more drastic hydrolysis conditions (4 N H_2SO_4 for 60 min at 121°) up to 90 per cent of the C^{14}-activity could be recovered in the nonvolatile fraction as free biotin. These discrepancies should be explained. In view of the fact that identical carboxylated active sites have been identified for propionyl carboxylase, methylmalonyl-oxalacetic transcarboxylase, and β-methylcrotonyl carboxylase, it seems unlikely that the site for acetyl carboxylase would be different.

Summary.—$C^{14}O_2$-enzyme was prepared by incubation of bovine liver mitochondrial propionyl carboxylase for several min with $HC^{14}O_3^-$, ATP, and Mg^{++}

and was isolated from the reaction mixture by gel filtration with Sephadex G-50. Carboxyl transfer from $C^{14}O_2$-enzyme to propionyl CoA to form methylmalonyl CoA was stoichiometric with the quantity of biotin present in the $C^{14}O_2$-enzyme (1 mole of carboxyl transferred per mole of biotin). $C^{14}O_2$-enzyme was methylated with CH_2N_2 and subjected to proteolytic digestion with Pronase (*Streptomyces griseus* protease). The fragment released, bearing essentially all of the radioactivity of the original $C^{14}O_2$-enzyme, was identified as N-carbomethoxybiocytin. The C^{14}-labeled N-carbomethoxybiocytin was hydrolyzed by the action of pig kidney biotinidase and the C^{14}-labeled hydrolysis product identified as 1′-N-carbomethoxy-(+)-biotin. Therefore, it is concluded that the carboxylated active site of propionyl carboxylase is ε-N-(1′-N-carboxy-(+)-biotinyl)-lysyl-enzyme.

We wish to thank Dr. J. Knappe for communicating the details for the assay and purification of biotinidase to us prior to their publication and also for providing a generous sample of 1′-N-carbomethoxybiocytin.

* Preceding paper in this series, Himes, R. H., D. L. Young, E. Ringelmann, and F. Lynen, *Biochem. Z.*, in press (1963).

† This paper represents the continuation of a series of investigations on bovine liver mitochondrial propionyl carboxylase. Following are references to the preceding papers in this series: *J. Biol. Chem.*, **235,** 878 (1960); *J. Biol. Chem.*, **235,** 3082 (1960); and *J. Biol. Chem.*, **237,** 2140 (1962).

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[2] Abbreviations: CoA, coenzyme A; ATP and ADP, adenosine 5′-triphosphate and diphosphate; Tris, tris (hydroxymethyl) amino methane; GSH, reduced glutathione; EDTA, ethylenediamine tetraacetate; DPNH, reduced diphosphopyridine nucleotide.

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*CHANGES IN THE PHYSICAL STATE OF DNA DURING THE REPLICATION CYCLE**

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Communicated by Renato Dulbecco, December 17, 1962

According to the original hypothesis of Watson and Crick[1] each strand of the DNA double helix serves as a template for the synthesis of a new complementary strand of opposite polarity. The observations of Meselson and Stahl[2] on the replication of DNA in *E. coli*, as well as the *in vitro* studies of Kornberg and co-workers,[3] have been entirely consistent with this hypothesis. However, studies with highly purified preparations of DNA polymerase have served to underscore a problem which was raised by Watson and Crick in their original formulation. (1) The strands of the DNA double helix are hydrogen bonded to each other and are not free to act as templates for replication. (2) DNA in its native double helical state is not an effective template for the synthesis of new DNA by highly purified preparations of polymerase.[4, 5]

Watson and Crick discussed the theoretical problem of the unwinding and separation of the strands. They suggested that unwinding might occur simultaneously with replication, synthesis starting at one end of a DNA molecule and continuing along its length. Our observations, on the contrary, suggest that the native helical configuration of DNA is altered at some time *prior* to replication, allowing the strands to act as templates for the synthesis of new DNA.

Observations on Satellite DNA Bands.—We have observed a satellite DNA band[6] of increased buoyant density and one of decreased buoyant density in studying nucleic acid preparations from exponentially growing bacteria at sedimentation equilibrium in CsCl.

(1) *Heavy satellite band:* A small satellite band with the buoyant density characteristic of denatured DNA is present in addition to the principal DNA band in the ultraviolet absorption photographs of Figure 1. We have observed this heavy satellite band on centrifugation of nucleic acid preparations[7] from exponentially growing[8] cultures of *E. coli*, *B. subtilis*, and *B. megaterium*.

Although the DNA of the satellite band resembles denatured DNA in its buoyant density and in its titration behavior in alkaline CsCl (Fig. 1, II *b*), it does not appear to be single stranded. This conclusion has been derived from density labeling experiments in which 5-bromouracil completely replaces thymine in one of the two conserved subunits[2] of DNA. Such half-substituted molecules are called "hybrid" DNA and have a density intermediate between unsubstituted and fully substituted molecules. Satellite bands have been observed corresponding to each of the three native DNA bands, unsubstituted, hybrid, and fully substituted. The satellite band corresponding to native hybrid DNA clearly does not contain single stranded DNA as this would be found either at the density of unsubstituted or fully substituted denatured DNA.

(2) *Light satellite band:* A satellite band of decreased buoyant density has been detected *within* the principle DNA band by ammonium sulfate fractionation of our

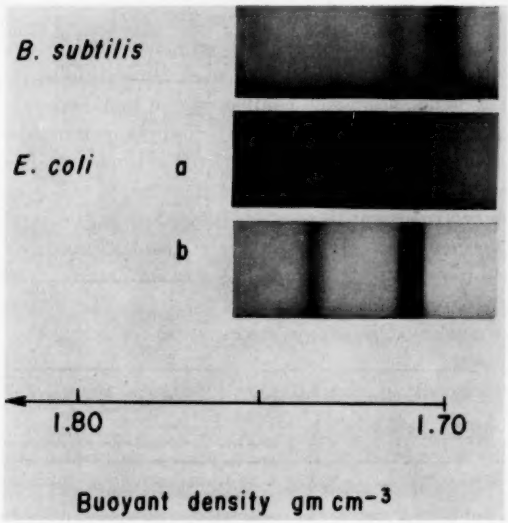

FIG. 1.—Ultraviolet absorption photographs of bands of bacterial DNA in 57.5 weight per cent CsCl solution after 24 hr of centrifugation at 44,770 rpm and 25°C. Bacteria were grown exponentially at 37°C in broth or in a supplemented glucose salts medium with aeration. Medium shifts were carried out using a coarse porosity collodion filter. Nucleic acids were isolated by a modified Kirby method[7] and conditions of ultracentrifugation and absorption photography are described elsewhere.[16] I. DNA isolated from an exponentially growing culture of *B. subtilis* II. (*a*) DNA from a culture of *E. coli* 15 T-A-U[9] starved for thymine for a period of three generations. A similar pattern is observed when DNA is obtained from exponentially growing cultures of this organism. (*b*) Sample as in II*a* but in CsCl solution buffered at pH 10.2 with 0.05 *M* phosphate. The observed increase in density of the satellite band is due to binding of Cs^+ by the polynucleotide, and indicates that the N1 protons of thymine and guanine are being titrated at the high pH. This effect has been described for denatured DNA but is not shown by native DNA.[17]

nucleic acid preparations. When one-half volume of saturated ammonium sulfate in 0.1 *M* sodium citrate is added to the nucleic acid preparation in the cold, a precipitate is formed which contains 10–20% of the total DNA of the preparation. Analytical density-gradient centrifugation of samples of the precipitate and supernatant reveals gross differences in the DNA band patterns of the two fractions. The pattern for the supernatant shows essentially only the principal DNA band. The pattern for the precipitate shows two satellite bands: (*a*) the satellite band of increased density is present as a minor component (heavy satellite band); (*b*) a very sharp band with a modal density slightly *less* (density difference 0.003 gm cm^{-3}) than that of the principal DNA band[10] is present as the major component (light satellite band). This ultraviolet absorption pattern corresponds closely with patterns of radioactive labeling which will be described below, suggesting that DNA which is identifiable by pulse radioactive labeling is precipitable under these conditions.

DNA Replication and the Satellite Bands.—Pulse radioactive labeling studies have shown that DNA molecules in the earliest stages of replication are found in at least two specific density regions. One of these regions corresponds to the denser satellite band, another is contained within the region of the principal DNA band and corresponds to the light satellite band. The experiments were carried out as follows, using

density labeling with 5-bromouracil and radioactive labeling to study DNA molecules at *different stages in their replication*. (1) Experiments were designed to label radioactively *only* those molecules which had initiated replication in the presence of the density label. The molecules which completed one replication during the labeling period are half-substituted with 5-bromouracil (hybrid) and of intermediate density. Those at earlier stages of replication contain smaller amounts of 5-bromouracil than hybrid molecules and have a smaller density increment. (2) Radioactive labeling was carried out for relatively short intervals (pulse labeling) comprising a small fraction of the time necessary to convert all of the unsubstituted DNA to hybrid. In this way we hoped to maximize the proportion of *incompletely* replicated molecules among those which were radioactively labeled. (3) The density distribution of radioactivity was examined to obtain information regarding the earliest stages in the replication process.

Figure 2 shows a density-gradient analysis of nucleic acids isolated from a culture

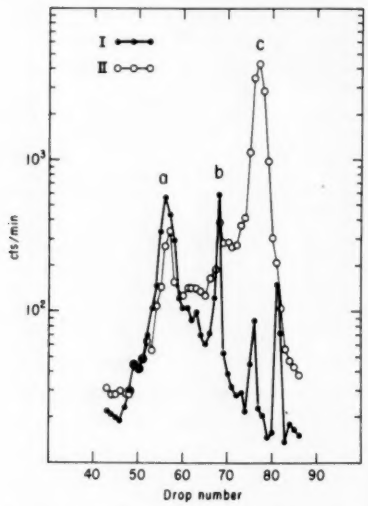

FIG. 2.—Analysis of density labeled and radioactively labeled DNA from *E. coli* 15 T-A-U. Bacteria were first grown exponentially at 20°C in the presence of 14.8 μC/μM (C^{14})-thymine for several generations, then were filtered, washed, and transferred to a 5-bromouracil containing medium. After 10 min $(P^{32}O_4)^{-3}$ was added to a specific activity of 10 μC/μM. After 20 min of P^{32} labeling growth was stopped with 0.01 *M* NaN_3 and the nucleic acids were isolated. For preparative density gradient centrifugation[18] a sample containing 2 μg of DNA in 0.5 ml CsCl solution of density 1.81 gm cm^{-3} was layered beneath 3 ml of CsCl solution (density 1.72 gm cm^{-3}, buffered at pH 8.6 with 0.02 *M* tris) in a lusteroid centrifuge tube. Density fractions were collected through a pinhole in the bottom of the tube after centrifugation for 50 hr at 36,000 rpm and 25°C. For assay of radioactivity in DNA, the fractions were incubated with 0.5 *M* KOH at 37°C for 24 hr to hydrolyse P^{32} labeled RNA to mononucleotides. Carrier calf thymus DNA (Worthington, 50 μg/ml) was added and the DNA was precipitated with cold 5 per cent trichloroacetic acid (TCA), collected on HA millipore filters, and washed 2× with cold 3 per cent TCA. The filters were dried and counted in the Tricarb Scintillation Spectrometer, utilizing the two channels (I, II) as follows. I. The distribution of P^{32} cpm in pulse labeled DNA. II. The distribution of C^{14} cpm in uniformly prelabeled DNA plus 0.35 times the activity in I. (*a*) Hybrid 5-bromouracil band. (*b*) Heavy satellite band. (*c*) Unsubstituted DNA band.

of *E. coli* 15 T-A-U[9] shortly after transfer from normal to 5-bromouracil containing medium. Only a small amount of DNA has been converted to hybrid (band *a*), about 10% is present in the heavy satellite band (band *b*), and the rest is found at the density of unsubstituted DNA (band *c*). Pulse labeled DNA synthesized shortly *after* the transfer is indicated by P^{32} activity which remains acid precipitable after the extensive alkaline treatment used to hydrolyse the labeled RNA. Its density distribution will be considered in some detail.

(1) *Pulse labeled DNA occurs in the region of the unsubstituted DNA band, but appears to be physically distinct from the bulk of the DNA of this band:* If pulse labeled DNA were physically associated with the bulk of the unsubstituted DNA, the density gradient distribution for the two kinds of DNA should not be grossly dis-

similar in the region of the unsubstituted DNA band. However, pulse labeled DNA occurs in two, distinct, narrow peaks within the DNA band and otherwise is essentially absent in this density region. The lightest of these two peaks corresponds in density to the mode of the light satellite band precipitable with ammonium sulfate. The narrowness of the peaks of pulse labeled DNA is consistent with the sharpness of the light satellite band observed after ammonium sulfate fractionation. It indicates that the labeled DNA has a significantly higher effective molecular weight in the CsCl gradient than the bulk of the DNA of the principal band. This could result from selective aggregation between pulse labeled DNA molecules of the same density, or might reflect a selective resistance to shear fragmentation of large chromosomal segments[11] which are pulse labeled. In accord with the latter possibility Goldstein and Brown[12] have demonstrated that pulse labeled DNA is selectively resistant to sonic fragmentation.

(2) *Pulse labeled DNA is physically associated with the DNA of the heavy satellite band:* In the region of the heavy satellite band the pulse labeled DNA concentration increases sharply, forming a large peak. The narrowness of this radioactive peak, like that of the peaks in the region of the unsubstituted DNA band, may be due either to selective aggregation or to shear resistance of the labeled molecules. There is a more gradual increase in the concentration of pulse labeled DNA between the position of the heavy satellite band and that of the hybrid DNA band, where a second large peak is found. In a number of experiments under conditions of high ionic strength ($\mu > 1$) and DNA concentration (> 20 μg/ml) a decrease of density of the heavy satellite band was observed, suggesting that the DNA of the satellite band readily "renatures."[13, 14] Under these conditions, when the DNA of the heavy satellite band has shifted to a region of lower density, the pulse labeled DNA shifts *in the same manner*.

(3) *The pulse labeled DNA in the region of the unsubstituted DNA band and the heavy satellite band consists of DNA molecules in early stages of replication:* Pulse labeled DNA molecules in late stages of replication would be expected to be found at or near the density of hybrid DNA, as all of the pulse labeled molecules initiated replication in the presence of 5-bromouracil. It might be that the finding of pulse labeled DNA in the region of the unsubstituted DNA band and heavy satellite band is an artifact, due to aggregation between pulse labeled hybrid DNA and unsubstituted DNA. This artifact is unlikely because the concentrations of DNA employed in density gradient analysis were very low (0.6 μg/ml) and because the radioactivity peaks do not correspond to positions of maximum DNA concentration in the density gradient. Two experimental observations support the conclusion that the pulse labeled DNA in the region of the unsubstituted DNA band and heavy satellite band consists of molecules in early stages of replication: (*a*) Pulse labeled DNA may be chased away from these regions by allowing further incorporation of 5-bromouracil in the absence of radioactive labeling. (*b*) Pulse labeled DNA is found selectively in the satellite band regions *even when the labeling is carried out in the absence of 5-bromouracil*.

Figure 3 illustrates the first of these two observations and shows the density region of the unsubstituted DNA band before (3*a*) and after (3*b*) a chase in nonradioactive, 5-bromouracil containing medium. Hybrid DNA bands have been omitted from the figure to facilitate comparison of the pulse labeled DNA distributions in the region

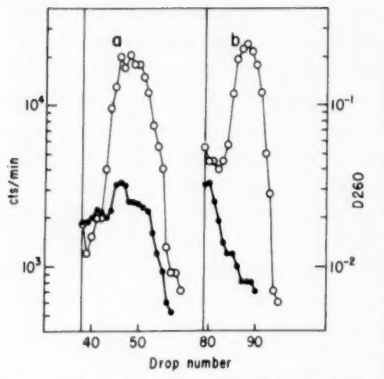

FIG. 3.—Chase of pulse P^{32} labeled DNA from the region of the unsubstituted DNA band by continued density labeling in a nonradioactive medium. (*a*) Density distribution of pulse P^{32} labeled DNA in the region of the unsubstituted DNA band before the chase with cold phosphorous. (*b*) Density distribution of pulse P^{32} labeled DNA in the same region as shown in (*a*) but after the chase with cold phosphorous. Exponentially growing bacteria (20° C, generation time 120 min) were filtered, washed, and resuspended in a 5-bromouracil containing medium for 15 min. $(P^{32}O_4)^{-3}$ was added to a specific activity of 100 μC/μM (4 μg/ml P) and an aliquot of culture was harvested after 30 min of radioactive labeling. A second aliquot was filtered, washed, resuspended in a nonradioactive 5-bromouracil containing medium with 200 μg/ml P, and harvested after 120 min in the nonradioactive medium. The nucleic acids were isolated from each sample, and treated with ribonuclease (Worthington, 10 μg/ml). After dialysis in the cold for 40 hr against 0.5 *M* NaCl, 0.015 *M* sodium citrate the nucleic acids were studied by preparative density gradient centrifugation. 80 μg DNA was used, and density fractions were plated on aluminum planchets, dried, and counted in a thin window gas counter (Nuclear Chicago) after dilution to a volume of 0.5 ml and measurement of absorption at 260 mμ (D260). ●, P^{32} cpm. ○, D260.

of interest. Higher DNA concentrations (20 μg/ml) were employed in this experiment and, as may be seen in Figure 3*a*, under these conditions the pulse labeled DNA of the heavy satellite band has renatured and is found to overlap the unsubstituted DNA band. After removal of the radioactivity, incorporation of 5-bromouracil was allowed to continue until one-third of the originally unsubstituted DNA was converted to hybrid. Figure 3*b* shows that more than 70 per cent of the pulse labeled DNA was displaced from the region of the unsubstituted DNA band by the further incorporation of 5-bromouracil.

The results of a pulse labeling experiment *not* involving 5-bromouracil substitution are shown in Figure 4. To achieve conditions of very limited DNA synthesis during pulse labeling, cells were thymine starved for a period which was too short to

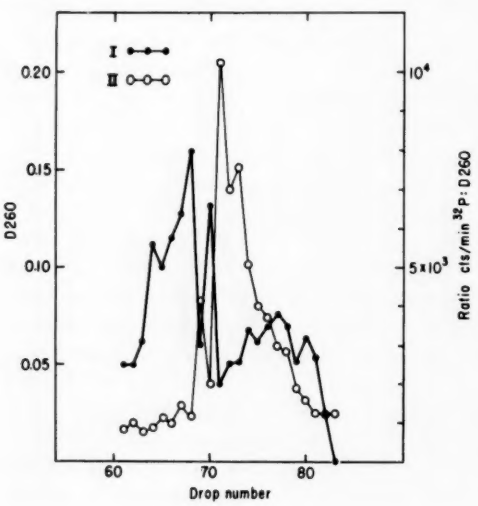

FIG. 4.—Density gradient analysis of DNA from *E. coli* 15 T-A-U labeled with $(P^{32}O_4)^{-3}$ in a thymineless medium. I. The ratio of cpm P^{32} above background to the optical density at 260 mμ. This is a lower bound for the specific activity of the DNA. II. Optical density at 260 mμ (D260). Bacteria growing exponentially at 20°C were filtered, washed, and resuspended in a thymineless medium and $(P^{32}O_4)^{-3}$ was added to 2 μC/μM. After 20 min growth was stopped and the bacterial nucleic acids were isolated. Ribonuclease (Worthington, 10 μg/ml) was added to the nucleic acid preparation, which was then dialysed 40 hr against 0.5 *M* NaCl, 0.015 *M* sodium citrate in the cold. Preparative density gradient analysis was carried out as described for Figure 3 except that 20 μg DNA was used. Similar results were obtained when pulse labeling was carried out with a 100-sec supply of (C^{14})-thymine, instead of P^{32}.

allow any loss of viability. After labeling, density gradient analysis revealed that the DNA of highest specific activity was found in a region of increased density, corresponding to the heavy satellite band, and in a second region on the light side of the main DNA band, corresponding to the light satellite band. When the pH of the banding solution was increased to 10.2, high specific activity DNA extended to regions of even greater density, consistent with the expected shift of the heavy satellite band at the higher pH. Experiments of this kind are in agreement with the studies described above; they confirm the specific changes in buoyant density found for actively replicating DNA molecules.

Discussion.—From the work of Meselson and Stahl[2] it is known that few molecules of the total DNA complement of *E. coli* are actively replicating at any given time during the division cycle. We have presented evidence suggesting that a significant proportion of the total DNA of *E. coli* may be isolated in states differing from native DNA in buoyant density and that *molecules in early stages of replication are found in these states rather than in the "native" state.* Taken together, these observations suggest that preparatory to replication DNA enters a state in which it differs structurally from native DNA. It is tempting to try to infer some of the characteristics of this *in vivo* state from the properties of the DNA after isolation.

After density gradient centrifugation, two DNA components of unusual density are found. The component which has been more extensively studied forms a distinct band of increased density relative to the bulk of the DNA (heavy satellite band). These experiments suggest that it is a collapsed structure in which the conserved subunits are not dissociated. The other component is found at a position overlapping that of the bulk of the DNA, but at a slightly reduced density (light satellite band). Experiments in progress to characterize the latter component have indicated that it is unstable and subject to low recovery during density-gradient centrifugation. Its reduced density and its precipitability with ammonium sulfate suggest that it may be associated with specific proteins, possibly polymerases[15] and nucleases. It is possible that the observed differences between these two unusual component DNA's are merely a consequence of the isolation procedure. Conversely, they may reflect significant differences in stability between different classes of DNA molecules in a "prereplication" state.

The collapse of the component which gives rise to the heavy satellite band suggests that it does not have the ordered helical structure postulated by Watson and Crick. This low stability might be brought about by a reduction of the base-base Van-der-Walls interactions or by an interference with the hydrogen bonding between the strands. In any case, the strands of such a destabilized structure would be available to act as templates more readily than would the strands of "native" DNA.

We wish to acknowledge the kind interest and helpful criticism of Professor Renato Dulbecco. P. C. Hanawalt and J. D. Smith provided helpful discussions.

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[6] Other workers have also observed satellite bands in studying DNA from a variety of sources. Ephrussi-Taylor (*J. de Chim. Phys.*, **58,** 1090 (1961)) working with synchronized *D. pneumoniae*, and Lark, Cavalieri, and Rosenberg (unpublished observations, 1962) studying synchronized *A. faecalis*, have observed satellite bands of increased buoyant density. With animal cell DNA, satellite bands both of increased and of decreased density have been reported (Kit, S., *J. Mol. Biol.*, **3,** 711 (1961); Sueoka, N., *J. Mol. Biol.*, **3,** 31 (1961); Schildkraut, C., J. Marmur, and P. Doty, *J. Mol. Biol.*, **4,** 430 (1962)). Bands of decreased density were also reported by Marmur, Rownd, Falkow, Baron, Schildkraut, and Doty (these PROCEEDINGS, **47,** 972 (1961)) with purified DNA from *A. faecalis* and *S. marcesens* following preparative CsCl-density-gradient fractionation.

[7] When bacterial DNA is isolated by other standard procedures (e.g., Marmur, J., *J. Mol. Biol.*, **3,** 208 (1961)) or by density-gradient centrifugation of bacterial lysates[2] the satellite band is not observed. Our isolation procedure utilizes deproteinization with phenol (Kirby, K. S., *Biochem. J.*, **66,** 495 (1957)) and ammonium sulfate-isopropanol precipitation of the nucleic acids. It will be described in detail elsewhere. The heavy satellite band is not an artifact of preparation, as it has been verified experimentally that native DNA is not denatured by our isolation procedure.

[8] With saturated cultures, or cultures in which DNA synthesis has run to completion after a shift to a medium lacking an essential amino acid (or after a shift from a medium containing amino acids to a minimal medium),[9] the satellite band is absent or greatly reduced in amount. However, if DNA synthesis in *E. coli* 15 T-A-U is stopped by shifting an exponentially growing culture to a thymineless medium the satellite band is observed to be undiminished (Fig. 1).

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*THE EFFECT OF SECONDARY STRUCTURE ON THE TEMPLATE ACTIVITY OF POLYRIBONUCLEOTIDES**

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Current experiments on the synthesis of polynucleotides and proteins seem to us to emphasize the functional importance of polynucleotide secondary structure. Thus, for example, the highly ordered double-helical structure of DNA[1] readily leads to ideas concerning DNA replication.[2, 3] Although our notions concerning the

secondary structure of the various types of RNA are much less precise, it does appear that RNA molecules are primarily single stranded and contain varying degrees of helical content.[4, 5] Recently, investigators from two different laboratories have proposed similar helical structures for transfer-RNA[6, 7] and both groups have discussed the functional significance of the suggested configurations. It is therefore of interest to consider the secondary structure of template RNA. Previous reports from this laboratory[8, 9] described the template RNA dependent incorporation of amino acids into proteins in a stable cell-free system from *E. coli*. Using this system and randomly mixed polyribonucleotides composed of various combinations of the four common ribonucleotides, nucleotide codewords corresponding to almost all of the protein amino acids have been determined.[10–15] Observations made with this system suggested that the secondary structure of polymers influenced template efficiency. For example, the ability of poly U to direct polyphenylalanine synthesis is lost when poly A-poly U double or triple helices are formed.[9, 10]

The present report describes certain physical properties of a series of poly UG preparations as well as the efficiency of these polymers in directing amino acid incorporation in the cell-free system. The data indicate that secondary structure in a polyribonucleotide limits its efficiency as a template. This finding is discussed and a specific functional role for polynucleotide secondary structure in the coding mechanism is proposed.

A preliminary account of some of these data has been published.[10]

Materials and Methods.—The procedures for the synthesis of polymers, determination of base ratios in the polymers, and measurement of amino acid incorporation have been described previously.[9, 14] The UG polymers are isolated by a modified procedure[16] designed to concentrate the longer chain length polymer and eliminate some of the shorter chain material. At the end of the polymerization the reaction mixture is deproteinized by the method of Sevag.[17] The aqueous solution of polymer is made 2 *M* in KCl by addition of an appropriate amount of solid KCl. Polymer is precipitated by the addition of 0.2 volume of cold absolute alcohol, and collected by centrifugation. The precipitate is dissolved in a small amount of water. In several cases the polymers did not dissolve readily in water unless a small amount of EDTA (final concentration about 5 mM) was added. Precipitation with KCl and alcohol is repeated two more times. The final precipitate is washed successively with 80%, 95%, and 100% alcohol and finally with ether and dried over paraffin. Polymers were subsequently dissolved and dialyzed against distilled water before use.

The phosphorolysis of the polymers by polynucleotide phosphorylase was determined by measuring the formation of P^{32}-labeled nucleoside diphosphate in the presence of P_i^{32}. The procedure referred to as Assay A by Singer and Guss[18] was used; the pertinent polymer was substituted for the poly A of that method. For the phosphorolysis studies, *Micrococcus lysodeikticus* polynucleotide phosphorylase, purified approximately 250-fold (Fraction VIII[19]) was used.

Measurements of the temperature dependence of the spectra of polymers ("melting curves") were carried out either in a Cary recording, model 14M, or Beckman, model DU, spectrophotometer. The Cary instrument was equipped with a thermostatted cell holder and the temperature was measured inside the sealed quartz cuvette by means of a hypodermic needle type thermistor. The Beckman instrument was equipped with thermospacers and the temperature was estimated in a water-filled, unsealed, blank cuvette. All solutions were gassed with helium just before filling the cuvettes and the cuvettes were sealed with a coating of General Electric Company RTV–60, silicone rubber compound.[20]

The chain lengths of polymers were determined by measuring the ratio of total organic phosphate to phosphate removable by *E. coli* alkaline phosphatase (Worthington Biochemical Corporation). The procedure outlined by Heppel and co-workers[21] was followed.

Sedimentation velocity studies were performed at 56,100 rpm and 20°C using the Spinco

Model E Ultracentrifuge equipped with UV absorption optics. The solutions contained approximately 0.03 mg of polymer per ml of 0.05 *M* cacodylate and were 0.1 *M* in NaCl. For measurements at neutral pH the cacodylate served as a buffer at pH 7.2; for alkaline measurements the solutions were made 0.01 *M* in KOH (pH 12). Tracings were obtained from photographic images of the cell using the Joyce-Loeble Recording Microdensitometer. The sedimentation coefficient was calculated[22] from the rate of change of position of the 50% point of the boundary.

Optical rotation was measured on a Rudolph Polarimeter using the mercury line at 365 mμ and a 1 decimeter light path.

Results.—Dependence of amino acid incorporation on the base ratio in poly UG: Table 1 shows the incorporation of phenylalanine, valine, leucine, and tryptophan into protein, measured with a series of poly UG preparations of increasing G content. The relative amounts of the four amino acids incorporated with any particular polymer are clearly related to the proportion of U and G. The data show that efficient incorporation of tryptophan requires a greater proportion of G in the polymer than is necessary for incorporation of valine or leucine. These data, therefore, confirm the earlier assignment of codewords UUG, UUG, and UGG to valine, leucine, and tryptophan, respectively.[11, 12, 14]

It is evident that the efficiency of a polymer as template RNA (Table 1) for any of the amino acids shown decreases sharply when the U/G ratio becomes less than 1. For example, the incorporation of phenylalanine with the polymer of U/G ratio 6.7/1 is about 60-fold greater than that obtained with the polymer of U/G ratio, 0.58/1. Although the decreased ability of the latter polymer to direct phenylalanine incorporation is expected, the lack of activity in coding for leucine, valine, and tryptophan is surprising. In an attempt to understand this observation various properties of the polymers were investigated and the results of these studies are presented below.

Chain length of polymers: Each of the polymers shown in Table 1 had a chain length in excess of 300 nucleotide units but probably not greater than 500 units. Because of the large amounts of polymer required for the chain length determination, more accurate measurements were not made.

Sedimentation characteristics of polymers: The sedimentation coefficients of the polymers described in Table 1 are given in Table 2. At pH 7.2 the S_{20} of the poly-

TABLE 1

STIMULATION OF C^{14} AMINO ACID INCORPORATION BY POLY UG

Polymer base ratio (U/G)*	6.7/1	3.3/1	1.6/1	0.58/1	
Nucleoside diphosphate ratio (U/G)†	8/1	5/1	3/1	1/1	Control minus polynucleotide
	Incorporation above Control‡ μμmoles				
C^{14} amino acid					
Phenylalanine	901	1708	1067	15	45
Valine	287	1036	1050	61	13
Leucine	267	834	856	35	64
Tryptophan	38	210	276	10	60

* Base ratio of polymer determined as previously described.[14]

† Ratio of UDP to GDP used in polymer synthesis.

‡ Figures represent incorporation of C^{14} amino acid above the basal incorporation obtained in the absence of added polymer. Basal incorporation is given in the last column.

Reaction mixtures (0.5 ml) contained 0.1 *M* Tris buffer, pH 7.8, 0.01 *M* magnesium acetate; 0.05 *M* KCl; 6×10^{-3} *M* β-mercaptoethanol; 1×10^{-3} *M* ATP; 5×10^{-3} *M* potassium phosphoenolpyruvate; 10 μg crystalline phosphoenolpyruvate kinase (CalBiochem); 2×10^{-4} *M* of each of 19 L-amino acids lacking the C^{14} amino acid; 0.8×10^{-4} *M* C^{14} amino acid; 5 μg of polynucleotide; and preincubated, dialyzed S–30 *E. coli* extract.[9] Each assay was performed in duplicate. The specific radioactivities of the amino acids varied 2–6 millicuries per millimole. All reaction mixtures were incubated at 37° for 90 min. These incorporation data therefore represent total incorporation of C^{14}-amino acids into protein rather than rate of incorporation.

TABLE 2

SEDIMENTATION COEFFICIENTS OF POLY UG PREPARATIONS

Polymer base ratio (U/G)	S_{20}*	
	pH 7.2	pH 12
6.7/1	5.9	5.2
3.3/1	3.1	2.3
1.6/1	5.7	3.3
0.58/1	11.0	2.1

* Inspection of the densitometer tracings indicated relatively little or no breakdown of the polymers during sedimentation at pH 12. Nonsedimenting material amounted to approximately 5%, at pH 7 and 12, with all the polymers except the last (U/G, 0.58/1), when it was about 20% at pH 12. Furthermore, in each case the sedimenting material was more homogeneous at pH 12 than at pH 7 and this was most striking with the last polymer (U/G, 0.58/1).

mer containing 70 per cent G is considerably higher than the S_{20} values of the others. However, the decrease in S_{20} noted for all polymers at pH 12 is much greater for this high G containing polymer.

Phosphorolysis of polymers by polynucleotide phosphorylase: The susceptibility of the poly UG preparations to phosphorolytic cleavage by *M. lysodeikticus* polynucleotide phosphorylase was studied and the results are presented in Table 3.

TABLE 3

PHOSPHOROLYSIS OF POLY UG BY POLYNUCLEOTIDE PHOSPHORYLASE

Base Ratio (U/G)	Phosphorolysis Rate (mμmoles/15 min.)	Phosphorolysis Rate† / Rate with poly A
poly U*	...	6.4
6.7/1	22.6	3.2
3.3/1	25.9	3.7
1.6/1	11.6	1.7
0.58/1	1.1	0.16

* Experiment carried out at a separate time.
† The numbers in the last column represent the rate of phosphorolysis of the given polymer relative to that of poly A.
The incubation mixtures contained approximately one μmole of polymer phosphate per ml (see section on *Materials and Methods* for details).

The rate of phosphorolysis (estimated with a measurement at a single time when the reaction rate is known to be linear with homopolymers) depends on the relative content of uracil and guanine. In particular, when the polymer has more guanine than uracil the rate falls off very sharply. This effect is not the result of the decreasing uracil content since the members of an analogous series of poly UC preparations were all phosphorolyzed at similar rates. Thus, when the ratio of uracil to cytosine was 4/1, 3.3/1, 1.7/1, and 0.6/1, the rates of phosphorolysis relative to poly A were 3.3, 4.0, 3.7, and 2.2, respectively. The slow phosphorolysis of poly UG containing more guanine than uracil appears to result from the high guanine content.

Effect of temperature on the ultraviolet absorption spectra of poly UG preparations: Thomas[23] and Doty and co-workers[4, 5] have discussed the use of hypochromicity as an indicator of secondary structure in polynucleotides. We have determined so-called "melting curves" for polymers described in Table 1. Polymers having ratios of uracil to guanine greater than 2/1 showed essentially no change in absorption between 200 and 300 mμ between room temperature and 85°. With poly UG having a uracil to guanine ratio of 1.6 to 1, we observed a slow increase in the absorption at 250, 260, 270, and 280 mμ upon increasing the temperature from 22° to 82°; however, the total increase at 270 mμ was only about 5 per cent (Fig. 1).

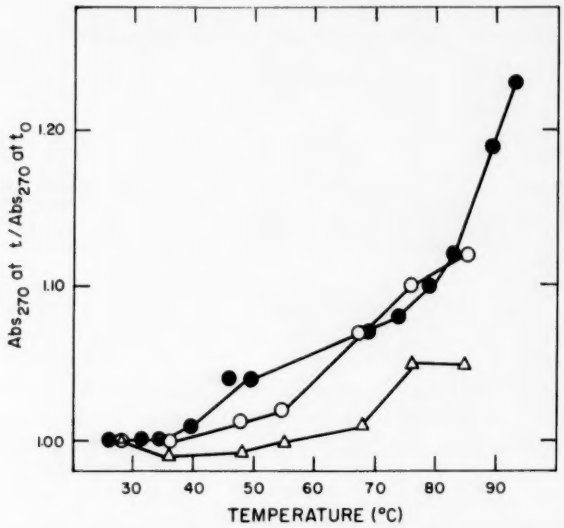

FIG. 1.—Changes in ultraviolet absorption of poly UG as a function of temperature. The ordinate is the ratio between the absorption at 270 mμ at the indicated temperature and the absorption at 270 mμ at the first temperature measured. —●—, poly UG, U/G ratio equal to 0.58/1, in 0.1 *M* KCl, pH 7.8; —○— poly UG, U/G ratio equal to 0.58/1, in 0.01 *M* potassium phosphate, pH 7.0; —△—, poly UG, U/G ratio equal to 1.6/1 in 0.01 *M* potassium phosphate, pH 7.0.

Under the same conditions (0.01 *M* phosphate buffer) poly UG having a uracil to guanine ratio of 0.58 to 1 gave a total increase in absorption at 270 mμ of 12 per cent on raising the temperature from 30° to 85° (Fig. 1). The data are given for 270 mμ since the hypochromicity of G containing structures appears to be optimal at that wavelength.[24] Figure 1 shows two curves for poly UG (U/G, 0.58/1); one was obtained in 0.1 *M* KCl, pH 7.8, the other in 0.01 *M* phosphate buffer, pH 7.0. The curves are similar although the start in the increase in absorption is somewhat delayed in 0.01 *M* phosphate buffer, pH 7.0. This observation is consistent with the finding[25] that the "T_m" of d-pGpGpGpG is lower in 0.2 *M* NaCl than in phosphate buffer alone. The experiment in 0.1 *M* KCl was carried out in the Cary instrument and a temperature dependent shift in the wavelength of maximum absorption from 255.5 mμ to 253 mμ was also observed. This shift took place at about 90°.

Optical rotation of poly UG preparations: As an independent measure of polynucleotide helical content[4] the optical rotations of several polymers shown in Table 1 were measured. Determinations were carried out at room temperature in 0.01 *M* potassium phosphate buffer, pH 7.0, using the mercury line at 365 mμ. The specific rotations of the polymers having U/G ratios of 6.7/1, 3.3/1, and 0.58/1, were +99, +74, and +399, respectively.

Discussion.—Amino acid incorporation into protein: The relative amounts of phenylalanine, valine, leucine, and tryptophan directed into protein by the poly UG preparations (Table 1) is clearly related to the base ratio of the polymer and

these data provide strong support for earlier codeword assignments.[11, 12, 14] However, several properties of the poly UG preparations used indicate that meaningful comparisons between theoretical frequencies of doublets or triplets and relative amino acid incorporations cannot be made. One such factor is the secondary structure of the polymers and is discussed in detail below. Another factor is the possibility that the polymers may be nonrandom. Since G is incorporated into polymer preferentially (Table 1) the ratio of UDP to GDP changes during polymer synthesis. Thus, the base ratio determined by analysis could represent the average base ratio rather than that of any particular polynucleotide region or molecule. Preferential incorporation of G into polymers has been noted previously,[14, 15] and similar observations have been made by Bretscher and Grunberg-Manago,[26] using *A. agilis* polynucleotide phosphorylase. The mechanism of this concentration phenomenon is currently under investigation.[27]

Effect of secondary structure on the template activity of messenger RNA: Several lines of evidence presented above indicate that copolymers of U and G contain a high degree of secondary structure when the relative content of G is high, and this interpretation is consistent with recent reports on the secondary structure of poly G itself.[24, 25, 28–30] (1) Although the four UG preparations described have approximately the same chain lengths the sedimentation coefficient of poly UG (0.58/1) at neutral pH is markedly higher than that of the others. This increase in S value may result from greater aggregation (due to hydrogen bonding) with increasing G content for, at pH 12, where the secondary structure of poly G collapses,[25, 28] all the polymers have similar sedimentation characteristics. (2) The sharp drop in phosphorolysis rate observed on going from a poly UG with a U/G ratio of 1.6/1 to one with a U/G ratio of 0.58/1 can also be explained by an increase in secondary structure. Ochoa[31] and Grunberg-Manago[32] have shown that polyribonucleotides having ordered secondary structures are phosphorolyzed much less rapidly than polymers existing as random coils. Poly G, for example, is completely resistant to phosphorolysis by polynucleotide phosphorylase.[19, 32] (3) Only polymers containing relatively large amounts of G (U/G, 1.6/1, and 0.58/1) showed any increase in ultraviolet absorption at raised temperatures, and the increase is greater the higher the G content. (4) The relatively high optical rotation of poly UG (U/G, 0.58/1) also indicates ordered secondary structure.[4]

Thus, the first three polynucleotides listed in Table 1 contain only moderate amounts of secondary structure and direct amino acid into protein with high efficiency. The last polymer, which contains almost 70 per cent G, exhibits a great deal of secondary structure and a markedly decreased ability to serve as a template for protein synthesis. This correlation between template efficiency and secondary structure is consistent with our earlier observation that the ability of poly U to direct polyphenylalanine synthesis is lost when poly A-poly U helices are formed.[9] Furthermore, we have found[10] that the extent of inhibition of polyphenylalanine synthesis by oligoadenylic acid preparations increases with the length of the oligonucleotide chain and can be correlated with the stability of oligoadenylic-poly U helices.

Thus, single strandedness and lack of extensive intramolecular hydrogen bonding appear to be requisite for messenger RNA activity in this *in vitro* system. Results obtained with natural RNA preparations are in accord with this conclusion. For

example, TMV-RNA directs protein synthesis with high efficiency[9, 34] in this system, compared to the efficiency of other RNA preparations.[9] Melting curves for TMV-RNA[4, 5] indicate that a larger per cent of its secondary structure is destroyed at 37° than is destroyed with ribosomal or transfer RNA preparations.

From an experimental point of view, caution should be used in handling template RNA to be used in *in vitro* systems. In the course of experiments on the melting of polymers we noted that the change in absorption with temperature is not always readily reversible. Thus the storage (for example, freezing and thawing) of polymer solutions may cause significant changes in secondary structure and therefore in template efficiency.

At present three factors influencing template activity are known—the size of the polymer chain,[14, 15, 33, 35] the nucleotide sequence, and the secondary structure. Previously we showed that poly U fractions of relatively high molecular weight are more active as templates than smaller molecules[14] and more recent data indicate that high template activity corresponds to average chain lengths of 100 or greater.[33, 35] The effect of nucleotide sequence on template efficiency is difficult to assess. Recent data show that the code is very degenerate[15] but it is not known whether all nucleotide sequences will code. Although nonsense sequences may exist, thus far none have been definitively demonstrated. As indicated in the present report, the secondary structure of a polynucleotide must also be considered in any evaluation of its ability to serve as template RNA in the *in vitro* system.

The molecular basis for the genetic information specifying the beginning or end of a protein is unknown. Previously we suggested that nonsense nucleotide sequences might function in this manner.[14] The results of the present study suggest an alternative explanation. Thus, a limited region of messenger RNA containing a high degree of secondary structure as a result of intramolecular hydrogen bonding (for example, G-G interaction) might also specify the beginning or end of a protein. Areas of marked secondary structure, which may be likened to knots in a rope, might separate the messenger molecules containing information for the synthesis of more than one protein into functional units. Mechanisms enabling certain proteins to be synthesized in close geographic proximity may be advantageous as, for example, in the synthesis of proteins containing different subunits[36, 37] or for the synthesis of coordinately repressed enzymes. In a more general sense, we expect that future studies of the three distinct RNA fractions, transfer RNA, ribosomal RNA, and messenger RNA, will indicate functional significance for the specific conformational aspects of their structures. With regard to messenger RNA, we would suggest that both the nucleotide sequence and secondary structure are relevant to the decipherment of the genetic code.

Summary.—A series of copolymers containing varying amounts of uracil and guanine have been studied as templates in a cell-free system for protein synthesis. Polymers containing relatively large proportions of guanine have low template activity. Evidence indicating that the guanine-rich polymers contain a high degree of ordered secondary structure is presented. It is suggested that the existence of such secondary structure accounts for the poor template activity of these polymers. The possible functional significance of RNA secondary structure in the coding mechanism is discussed.

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* The following abbreviations are used: Poly U, polyuridylic acid; poly A, polyadenylic acid; poly C, polycytidylic acid; poly G, polyguanylic acid; poly UC, copolymer of uridylic and cytidylic acids; poly UG, copolymer of uridylic and guanylic acids; G, guanylic acid; U, uridylic acid; A, adenylic acid; C, cytidylic acid; TMV, tobacco mosaic virus. All other abbreviations conform to those acceptable to the *Journal of Biological Chemistry*.

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*CHLORAMPHENICOL-PROMOTED REPRESSION OF β-GALACTOSIDASE SYNTHESIS IN ESCHERICHIA COLI**

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Chloramphenicol (CAP), an antibiotic which inhibits protein synthesis, has been shown to preferentially inhibit the synthesis of certain inducible enzymes in *Escherichia coli*.[1] This preferential inhibition occurs in levels of CAP which permit exponential growth at a reduced rate. There have been various reports which indicate that the synthesis of inducible proteins is more sensitive than total protein synthesis to the action of base analogues[2] and UV irradiation.[3, 4] The preferential susceptibility of induced enzyme synthesis to these agents suggests either of two basic mechanisms which might be involved: (*a*) induced enzymes are synthesized by a process involving some steps unlike those involved in the synthesis of noninduced proteins; or (*b*) certain inhibitors affect the regulatory mechanisms which control the synthesis of these proteins, in addition to affecting the steps of protein biosynthesis *per se*.

This report deals with experiments we have performed in an effort to distinguish between these two alternatives as they might apply to the preferential inhibition by CAP of β-galactosidase synthesis.

Materials and Methods.—1. *Bacterial cultures and media:* The following strains of *Escherichia coli* were employed: ML 30, inducible for β-galactosidase ($z^+i^+o^+$); ML 308, constitutive for β-galactosidase ($z^+i^-o^+$); K-12, 2000, partially constitutive for β-galactosidase ($z^+i^+o^c$); K-12, 200PS, F-lac$^+$, carrying episomal copies of the lac locus (z^+i^+). Experiments were usually conducted with organisms grown in a mineral salts medium containing the following in gm/liter: K_2HPO_4, 7; KH_2PO_4, 2; $(NH_4)_2SO_4$, 1; $MgSO_4$, $7H_2O$, 0.1; sodium citrate, 0.4. Glycerol (0.5%) was used as the carbon source.

2. *Biochemical analyses and enzymology:* Protein of bacterial cultures was assayed by the method of Lowry *et al.*[5] and ribonucleic acid by the orcinol method.[6] β-Galactosidase activity was determined by the hydrolysis of *o*-nitrophenyl-β-D-galactoside (ONPG) as described by Rickenberg *et al.*[7] The synthesis of β-galactosidase was induced by adding methyl-β-D-thiogalactoside (TMG) or isopropyl-β-D-thiogalactoside (IPTG) to a culture at a final concentration of 10^{-3}M. Enzyme assays with whole cells were performed by first treating the cell suspension with 0.05 ml toluene and 10 μg desoxycholate per ml suspension. The assays were conducted in 0.05 *M* sodium phosphate buffer, pH 7.0, which contained 1.0 μmole mercaptoethanol and 0.1 μmole $MnSO_4$ per ml.

3. *Inhibition studies:* Growth inhibitors were employed at concentrations which caused a 40–50% reduction in growth rates. All antibiotics and 5-methyltryptophan were studied in cultures growing at pH 7. However, sodium azide and 2,4-dinitrophenol were found to be optimally active in inhibiting growth at pH 6. There were no differences in growth rates of noninhibited cultures growing at either pH.

4. *Radioactivity determinations:* The incorporation of C^{14}-amino acids or C^{14}-pyrimidines into trichloracetic acid (TCA) insoluble material was determined by removing samples from the incubation mixtures and pipetting them into equal quantities of 10% TCA. These were allowed to stand in the cold for 15 min and were then poured onto a membrane filter (Millipore, HA). The residues were washed thoroughly with 5% TCA. The filters were removed from the holders and glued to planchets. Radioactivity measurements were made in a Nuclear-Chicago windowless, gas-flow counter.

5. *Preparation of antiserum:* Antiserum against β-galactosidase was prepared with an antigen of partially purified enzyme, obtained by ammonium sulfate fractionation as described by Cohn

and Monod.[8] Rabbits were immunized with the partially purified enzyme suspended in Freund's adjuvant (without the mycobacteria). Weekly injections of increasing amounts were given for eight weeks, then the rabbits bled and the sera pooled.

Results.—Effect of CAP on "constitutive" β-galactosidase synthesis: In a previous report we demonstrated that enzymes which are normally synthesized constitutively in *E. coli* are not preferentially affected by CAP.[1] We have determined the effect of this antibiotic on the constitutive synthesis of β-galactosidase in two genotypically different mutants (Fig. 1). The first mutant, $z^+o^+i^-$, is constitutive by virtue of a mutation in the gene which specifies the cytoplasmic repressor.[10] This mutant will synthesize β-galactosidase in the absence of inducer at the same rate as an induced, wild-type organism. The second constitutive mutant, $z^+o^ci^+$, is partially constitutive, and makes enzyme in the absence of inducer at about 20 per cent the induced rate. It can be seen in Figure 1*A* that the inhibition kinetics for the i^- (repressor) mutant are similar to those shown previously for the inducible organism.[1] However, the inhibition kinetics for the o^c (operator) mutant were markedly different from those of the i^- and inducible organisms (Fig. 1*B*). In the o^c mutant the preferential effect on enzyme synthesis of the antibiotic was not apparent until after a considerable lag, amounting to about 0.5 generations (or 100 min where the generation time was 200 min). It can be seen in Figure 1*A* that inducer has no effect on the inhibition by CAP of constitutive β-galactosidase synthesis in the i^- mutant.

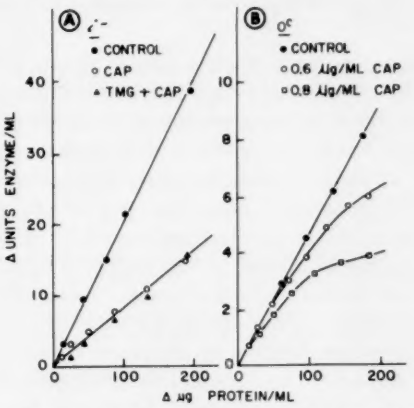

FIG. 1.—(*A*) Preferential inhibition by CAP of the constitutive synthesis of β-galactosidase in ML 308 (i^-). An exponentially growing culture was divided into 3 flasks. One culture became the control (nontreated), the second received 0.8 μg/ml CAP, and the third received 0.8 μg/ml CAP and TMG (10^{-3} Molar). Samples were removed from each culture at intervals during the ensuing growth, and enzyme and protein content determined. The values for enzyme and protein are expressed as the increase from the time the additions were made.

(*B*) Preferential inhibition by CAP of the constitutive synthesis of β-galactosidase in strain K-12; 2000 (o^c). Procedure was the same as in Figure 1*A*.

From these experiments it appears that the preferential inhibition of β-galactosidase synthesis involves the operator (*o*) gene but not necessarily the product of the regulator (*i*) gene. It is apparent, furthermore, that the state of the enzyme in the wild-type organism, i.e., inducible or constitutive, determines whether or not it will be preferentially sensitive to CAP.

The specificity of the preferential inhibition of β-galactosidase synthesis was determined by testing the effects on enzyme synthesis of various inhibitors. The studies were conducted with the i^- constitutive mutant (ML 308) since it responds to CAP in a manner similar to the inducible strain. In Table 1 it can be seen that the inhibition of growth rate alone is not sufficient to preferentially inhibit the synthesis of β-galactosidase, since 5-methyltryptophan, azide or DNP do not produce the effect. The preferential effect on enzyme synthesis was limited to the antibi-

TABLE 1

THE EFFECT ON β-GALACTOSIDASE SYNTHESIS OF VARIOUS METABOLIC INHIBITORS

Inhibitor*	Differential Rate† of Synthesis		
	Control	Inhibited	Decrease %
Tetracycline	0.080	0.050	38
Puromycin	0.080	0.055	32
Streptomycin	0.078	0.047	40
5-Methyltryptophan	0.081	0.081	0
Dinitrophenol	0.083	0.082	0
Sodium Azide	0.082	0.083	0

* Inhibitors were used at concentrations which reduced the growth rate to 40–50% the control.
† Final slope of the line resulting from a differential plot of Δ enzymes versus Δ protein.

otics, compounds which inhibit protein synthesis *per se*, but not nucleic acid synthesis. The results suggest that CAP does not preferentially inhibit β-galactosidase synthesis by some specific involvement of the antibiotic, but by a mechanism which may be elicited by other compounds.

Possible mechanisms for the preferential action of CAP on β-galactosidase synthesis: The preferential inhibition by CAP of β-galactosidase synthesis could be attributed to any of the following mechanisms: (*A*) cultures treated with CAP synthesize some enzyme as altered or enzymatically inactive protein; (*B*) CAP elicits the degradation of newly formed enzyme; (*C*) the site of action of CAP is more sensitive to the antibiotic in the case of inducible enzyme synthesis; or (*D*) the preferential inhibition results from an alteration by CAP of the regulatory mechanism for β-galactosidase. These possibilities are explored below.

A. *Synthesis of altered or inactive enzyme protein:* Several investigations have established that CAP normally inhibits protein synthesis by preventing the transfer of amino acids from charged s-RNA to ribosomes.[12] A partial block in this step, produced by the low CAP concentrations employed in this study, could result in the synthesis of small amounts of (*a*) altered or (*b*) inactive enzyme protein. Thus, the apparent preferential inhibition of β-galactosidase synthesis could be attributed to a portion of the total enzyme protein being enzymatically inactive, or lacking full enzymatic activity. To test (*a*), comparisons were made between the β-galactosidase of normal and that of CAP-treated bacteria. There were no differences between the enzymes with respect to pH optima, thermal inactivation, activation by metallic ions and reducing substances, and Michaelis-Menten constants. We conclude from these data that the β-galactosidase synthesized in CAP-grown bacteria is unaltered.

The possibility that CAP treatment results in the formation of enzymatically inactive protein ((*b*) above) was tested by assaying the extracts of CAP-grown bacteria for protein which is antigenically similar to β-galactosidase. The technique employed was that of Cohn and Perrin, described by Masters and Pardee.[13] Increasing amounts of extract were mixed with a constant quantity of antibody. After allowing time for the formation of the specific precipitate the mixtures were centrifuged. The amounts of enzyme remaining in the supernatant were determined and plotted against the quantities of enzyme added to the antiserum. If cross-reacting material (CRM) were present in the extract of CAP-treated bacteria, the point at which residual enzyme was detected in the supernatant would be shifted toward the origin. Figure 2 shows that there was no increased CRM formation in cells which had been grown in low levels of CAP. Therefore, the preferential

inhibition by CAP of β-galactosidase synthesis cannot be accounted for by the synthesis of inactive antigenically related protein.

B. *Does CAP elicit the degradation of β-galactosidase?* Rickenberg *et al.*[7] have shown that the synthesis of β-galactosidase ceases immediately after removal of inducer, and that the enzyme content per culture volume remains constant thereafter. The possibility that CAP activated some mechanism for degrading β-galactosidase was investigated by employing cultures undergoing "deadaptation." A culture of ML 30 was grown up in the presence of inducer, which was removed by filtering off the cells and resuspending them in medium lacking inducer. CAP was added and the cells were allowed to continue growing. Figure 3 shows that there was no loss of enzyme in the CAP-treated culture undergoing deadaptation. The theoretical value was calculated by assuming that the preferential effect of CAP was due to enzyme degradation. Under these conditions CAP does not elicit the degradation of β-galactosidase.

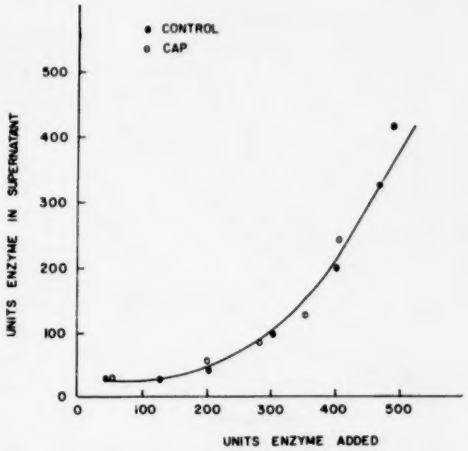

FIG. 2.—Immunological assay for cross-reacting material (CRM) in extracts from CAP-treated and nontreated bacteria. Increasing amounts of extract were added to constant volume of antiserum. After the formation of specific precipitates, the tubes were centrifuged and the supernatant removed and assayed for β-galactosidase remaining.

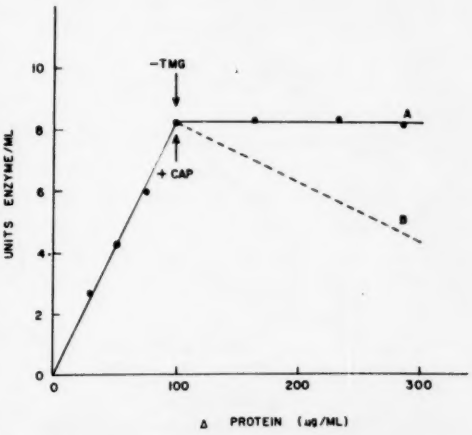

FIG. 3.—The persistence of β-galactosidase in a culture of ML 30 undergoing "deadaptation" in the presence of CAP. The dashed line (*B*) gives a calculated theoretical value, assuming that enzyme degradation can account for the preferential inhibition by CPA of β-galactosidase formation.

C. *Is the site of action of CAP more sensitive to the antibiotic in the case of β-galactosidase?* It is possible that the primary site of action of CAP is inherently more sensitive for the synthesis of β-galactosidase than for other proteins. If this is the case, the differential sensitivity should be exhibited under all conditions of enzyme and protein synthesis. To the contrary, Figure 4 shows that CAP does not preferentially affect β-galactosidase synthesis in cells depleted of both nitrogen and glycerol, although cells depleted of glycerol alone show the preferential inhibition.

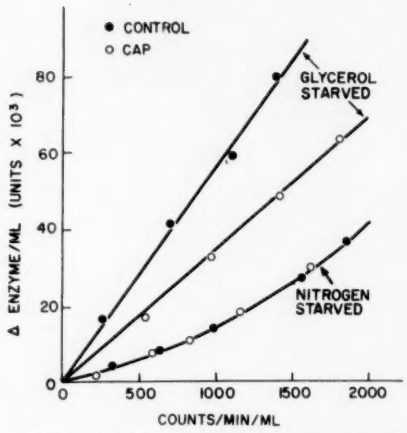

FIG. 4.—Effect of starvation on the inhibition by CAP of β-galactosidase synthesis. Cultures were depleted for either nitrogen and glycerol (lower curve) or glycerol alone (upper curves). The rate of leucine-C^{14} incorporation into TCA insoluble material was used as a measure of protein synthesis. The CAP treated suspension incorporated isotope at 55% of the rate of the nontreated suspensions.

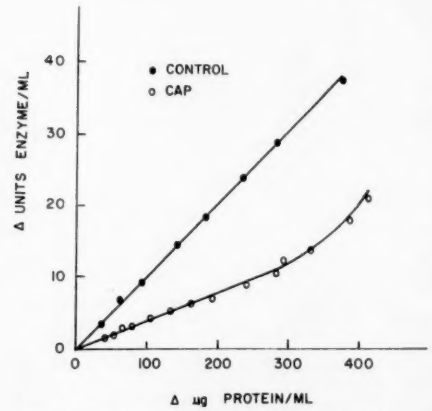

FIG. 5.—The "recovery" of the normal differential rate of β-galactosidase formation in a CAP-treated culture. Cultures of ML 30 were grown with inducer, in the presence and absence of CAP, for over three generations.

In addition, the preferential inhibition of β-galactosidase synthesis in a growing culture is lost after a long period of exposure even though growth remains inhibited. In the experiment shown in Figure 5, a fully induced culture of ML 30 was incubated with CAP for several generations and the levels of β-galactosidase were determined over the entire time. It can be seen that after about three generations of growth in CAP the differential rate of β-galactosidase synthesis recovered and reached the control rate. This recovery was not attributed to breakdown or intracellular dilution of CAP, since the growth rate remained inhibited. Furthermore, when cells which had recovered their normal differential rate of enzyme synthesis were diluted into fresh CAP-medium, there was no preferential inhibition of β-galactosidase synthesis. Upon subculture in medium lacking CAP, however, the same cells responded with a preferential inhibition of β-galactosidase synthesis when CAP was later added to the culture.

From these considerations, (C) has been eliminated as a possible mechanism for the preferential inhibition by CAP of β-galactosidase synthesis.

D. *The effect of CAP on the regulatory process:* The behavior of the o^c mutant toward CAP suggests that the antibiotic acts preferentially, and probably indirectly, through the operator gene of the *lac* operon. In the context of current concepts of the regulation of gene action,[9, 11, 15] an action on the operator gene could repress enzyme synthesis by inhibiting the initiation of messenger RNA synthesis. The inhibition of messenger RNA synthesis would occur in addition to the direct effect of CAP on protein synthesis *per se*, resulting in an additive effect on those enzymes which are regulated through an operator gene.

The hypothesis that CAP inhibits the synthesis of β-galactosidase messenger RNA was examined more directly by experiments similar to those of Jacob and Monod[9]

and Gros *et al.*[14] A culture of *E. coli* F-lac$^+$ was grown at 20°C with a generation time of about 4 hr. Exposure to uracil-C^{14} from 20 to 80 seconds yielded kinetics of isotope incorporation as shown in Figure 6, line *A*. Zone centrifugation analysis of the extracts showed that exposures to isotope of up to 60 seconds resulted in labeling of only the "messenger RNA" fraction. The addition of a β-galactosidase inducer (IPTG) 8 min prior to exposure to isotope resulted in an increase of about 22 per cent in the rate of isotope incorporation into RNA (Fig. 6, line *B*). This increase was referred to as the "inducer-promoted" increase. The effect of CAP alone on messenger RNA synthesis was determined by an 8 min incubation with CAP prior to the addition of uracil-C^{14}. These data are also shown in Figure 6 (line *C*). The stimulation by CAP of total messenger RNA synthesis was somewhat surprising. However, others have shown that the synthesis of ribosomal[16, 17] and "soluble" RNA[17] is stimulated by CAP. The increase in the rate of labeling of messenger RNA in the presence of CAP is referred to as the CAP-promoted increase.

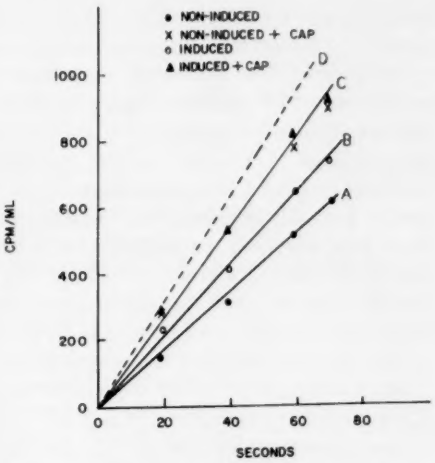

FIG. 6.—The rates of incorporation of uracil-C^{-14} into TCA insoluble material of F-lac$^+$ cultures under the conditions noted in the legend. A culture of the F-lac$^+$ organism, growing at 20°C, was divided into four portions, and the various additions were made. After 8 min incubation, uracil-C^{14} was added. Incorporation of isotope was stopped by aspirating the cells into an equal volume of 10% TCA. The dashed line gives the theoretical value expected if the CAP-promoted and inducer-promoted increases are additive.

It is now possible to make certain predictions concerning the effect on messenger RNA synthesis of CAP and inducer together. If CAP does not interfere with the synthesis of β-galactosidase messenger RNA (hence does not affect the induction process), the stimulation of isotope incorporation during the pulse will be the sum of the inducer-promoted and the CAP-promoted increases (that is, the two effects will be additive). If CAP does interfere with β-galactosidase messenger synthesis, there will be no detectable inducer-promoted increase in isotope incorporation over the CAP-promoted increase.

The effect of CAP and inducer on the rate of messenger RNA labeling is shown by line *D* of Figure 6. It can be seen that there was no detectable increase in the rate of labeling above the CAP-promoted increase. In these experiments, the CAP-promoted increase in labeling was not the maximum attainable rate, since it could be increased further by higher concentrations of CAP. Our interpretation of these results is that, although CAP treatment elicits the stimulation of total pulse-labeled RNA synthesis, it inhibits the synthesis of β-galactosidase messenger RNA. Furthermore, all the data are compatible with the conclusion that the action of CAP on β-galactosidase synthesis is the result of two additive mechanisms: the inhibition of protein synthesis at the ribosome level[12] and the inhibition of β-

galactosidase messenger RNA synthesis (induction). The latter effect would account for the preferential inhibition of β-galactosidase synthesis.

Discussion.—The following indirect evidence suggests that CAP alters the mechanism which regulates β-galactosidase synthesis: (*a*) We have found that only repressor-controlled enzymes are susceptible to the preferential action of CAP, (*b*) two enzymes in a single operon, β-galactosidase and galactoside permease, are inhibited by CAP to the same degree,[1] and (*c*) a mutant with a lesion in the operator gene is partially resistant to the preferential effect of CAP. Though suggestive, these data provided the rationale for a more direct examination of the possibility that CAP exerts its preferential effect on enzyme synthesis by inhibiting the induction process. For this examination, we employed the technique described by Jacob and Monod[11] and Gros *et al.*[14] for identifying an inducer-promoted increase in the rate of labeling of messenger RNA. Accordingly, it was found that in an F-lac$^+$ culture there was no detectable increase by inducer in the rate of messenger RNA labeling in the presence of CAP. Our interpretation is that CAP, by some indirect process, interferes with the induction process for β-galactosidase by inhibiting β-galactosidase messenger RNA synthesis.

The working hypothesis has been that the "repression" by CAP of β-galactosidase synthesis is the result of the accumulation of specific repressor molecules within the antibiotic-treated cells. Seemingly, a test of this hypothesis would be the failure of an i^- (constitutive) mutant to exhibit the preferential inhibition of β-galactosidase synthesis. However, as is shown in Figure 1, this mutant behaves in a similar fashion as the inducible, wild-type. Therefore, if the *i* gene controls the structure of the cytoplasmic repressor[10] and the i^- mutant fails to elaborate the repressor, the "repression" by CAP is not the result of the accumulation of *i* gene product. Alternatively, the i^- mutants we have examined could elaborate an altered or labile repressor, able to act only when the concentration reaches sufficient magnitude. In spite of the behavior of the i^- constitutive mutants, the repression effect of CAP seems to involve the operator gene, presumably by the mediation of some agent other than the *i* gene product. This is inferred from the inhibition kinetics of the o^c mutant in contrast to the kinetics of the i^- organism (Figs. 1*A* and 1*B*).

We have examined the possibility that the CAP promoted repression of β-galactosidase synthesis is due to catabolite repression (cf. Magasanik, ref. 18). The data indicate that catabolite repression is not the mechanism involved. These, and other data pertaining to the mechanism of CAP promoted repression, will appear in a subsequent communication.

Summary.—Chloramphenicol (CAP) inhibits the formation of β-galactosidase to a greater extent than the synthesis of total protein. We have presented evidence that CAP treatment results in the inhibition of the inducer-promoted synthesis of pulse-labeled RNA (mRNA). In the context of current concepts in molecular biology, these data support the conclusion that CAP inhibits the synthesis of β-galactosidase by two additive mechanisms: (*a*) inhibition of β-galactosidase messenger RNA synthesis, and (*b*) inhibition of the later steps in protein synthesis.

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† The data of this report were taken from a thesis submitted by the senior author to the Graduate School, Yale University, in partial fulfillment of the requirements for the degree of Doctor of

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*INDUCTION BY AN RNA PHAGE OF AN ENZYME CATALYZING INCORPORATION OF RIBONUCLEOTIDES INTO RIBONUCLEIC ACID**

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There is evidence that the replication of RNA[1] viruses, and therefore that of viral RNA, is not mediated by DNA whereas most, if not all, of the RNA synthesis of the host cells is DNA-controlled.[2, 3] It thus appears that the synthesis of viral RNA may be catalyzed by an RNA-primed enzyme.[2] Such an enzyme might be constitutive in the host cell or induced by infection with RNA viruses.

Evidence will be presented in this paper that *Escherichia coli* cells infected with an RNA phage contain an RNA-synthesizing enzyme which is not present, or is present in negligible amounts, in noninfected cells. The enzyme, which is particulate in nature, catalyzes the incorporation of nucleotides from ribonucleoside triphosphates into RNA. It has been purified about 50-fold. Optimal nucleotide incorporation requires the presence of all four ribonucleoside 5′-triphosphates, i.e., ATP, GTP, UTP, and CTP. The enzyme preparations thus far obtained contain RNA and show no dependence on added RNA for activity. To distinguish this enzyme from the DNA-dependent RNA polymerase, it will be referred to as RNA synthetase.

Baltimore and Franklin[4] have recently reported on the occurrence of a similar enzyme in L-cells infected with the RNA-containing Mengovirus.

Materials and Methods.—C^{14}-labeled ribonucleoside 5′-di- and triphosphates were obtained from Schwarz BioResearch, Inc., Orangeburg, N. Y. Nonlabeled ribonucleoside triphosphates were from Schwarz BioResearch, Inc. and the Pabst Laboratories, Milwaukee, Wisconsin. Calf thymus DNA, snake venom phosphodiesterase, and crystalline pancreatic RNAase and DNAase were obtained from the Worthington Biochemical Corporation, Freehold, New Jersey. Crystalline pyruvate kinase and phosphoenolpyruvate were from C. F. Boehringer and Sons, Mannheim, Germany. Actinomycin D was a gift of Dr. E. Alpert, Merck, Sharp and Dohme, Inc., West Point, Pennsylvania. Protamine sulfate was purchased from Sigma Chemical Company, St. Louis, Missouri.

Protein was determined by a modified biuret method[5] or by the method of Lowry *et al.*[6] Nucleic acid was determined spectrophotometrically[7] with use of the table given by Layne[8] to calculate the ratio of nucleic acid to protein.

Isolation of Enzyme.—Assay: The reaction mixtures contained (in μmoles): Tris HCl buffer, pH 7.2, 20; $MgCl_2$, 1.5; $MnCl_2$, 0.5, mercaptoethylamine, 2; phosphoenolpyruvate, 1; pyruvate kinase, 35 μg; DNAase, 25 μg; GTP-8-C^{14} (2.5 to 5 $\times$ 10^5 cpm/μmole), 0.1; ATP, CTP, and UTP, each 0.1; and enzyme in a final volume of 0.2 ml. A zero time control was run simultaneously with each sample. In this case GTP-C^{14} was omitted from the incubation mixture and added only after incubation.

Phosphoenolpyruvate and pyruvate kinase were used in the assay to avoid the formation of nucleoside 5′-diphosphates during incubation thus ensuring that any incorporation of nucleotides into RNA would not be due to the action of polynucleotide phosphorylase, an enzyme that catalyzes synthesis of polyribonucleotides from ribonucleoside 5′-diphosphates. That the bulk of the nucleotide incorporation observed could not be due to polynucleotide phosphorylase was further shown by the fact that 0.1 *M* orthophosphate, which would completely inhibit the synthetic activity of this enzyme, caused only 15–20 per cent inhibition of nucleotide incorporation by protoplast lysates of infected *E. coli* under the above assay conditions. This small inhibition may be due to precipitation of Mn^{++} by the phosphate. DNAase was present in the incubation mixture to destroy DNA and prevent the activity of RNA polymerase, RNA being a very poor primer of this enzyme.[9] Use of GTP as the labeled nucleotide avoided incorporation of label through the action of the enzyme catalyzing the synthesis of polyadenylic acid from ATP[10, 11] or through the enzyme catalyzing the addition of cytidylic and adenylic acid residues to partially degraded amino acid transfer RNA.[12, 13] After incubation for 10 min at 37°, 0.05 ml of eight per cent, neutralized sodium pyrophosphate and 10 ml of ice cold six per cent trichloroacetic acid were added to each sample. If necessary, 0.05 ml of one per cent crystalline bovine serum albumin was added to obtain bulky precipitation with trichloroacetic acid. The precipitate was collected by centrifugation at 2°, washed twice with 10 ml of six per cent trichloroacetic acid, dissolved in 1 ml of 1.0 *N* NH_4OH, plated, dried under an infrared lamp, and its radioactivity measured with use of a windowless Geiger-Müller counter.

Under the assay conditions, the rate of incorporation of GMP-C^{14} was linear up to 10 min and, with lysates of infected protoplasts, proportional to the amount of protein between 100 and 600 μg.

Enzyme unit: One unit was taken as the amount of enzyme catalyzing the in-

corporation of 1.0 mμmole of GMP-C^{14}/min and the specific activity expressed as units/mg protein.

Extraction: The RNA phage MS-2 used in this work, similar to Loeb and Zinders' f_2 coliphage,[14] and a culture of its host, *E. coli* Hfr 3,000, were kindly provided by Dr. A. J. Clark, Department of Microbiology, University of California, Berkeley. The cells were grown as described by Loeb and Zinder.[14] Cells were infected by adding approximately 10^{13} plaque-forming units of virus to one liter of culture during exponential growth and the infected cells were harvested by centrifugation 40 min later. Infected protoplasts were obtained by adding a concentrated solution of 200 gm of sucrose, 2 gm of $MgSO_4$ and 500,000 units of penicillin to one liter of culture during exponential growth (about 2×10^8 cells/ml) and infecting at a multiplicity of 50–100 thirty minutes later. The protoplasts were harvested by centrifugation one hour after infection. Absence of phage from cells used as noninfected controls was established by plating[15] at the time of harvesting.

Enzyme activity could be extracted with 0.005 *M* Tris HCl buffer, pH 7.2, containing 0.005 *M* mercaptoethylamine, from infected cells disrupted at 0°–5° by grinding with alumina, sonic oscillation, or homogenization with glass beads in a Waring blendor. However, higher yields were obtained from lysates of infected protoplasts and this was the method used routinely for extraction of the enzyme. The harvested protoplasts were lysed by stirring with 20 ml of 0.005 *M* Tris HCl buffer, pH 7.2, containing 0.005 *M* mercaptoethylamine, per liter of original culture. The viscous lysate was centrifuged for 20 min at 30,000 *g*. This yielded a supernatant and a highly viscous sediment which included cellular debris and some intact protoplasts and cells. Incubation of a suspension of this sediment with 5 μg of DNAase and 0.5 μmole of $MgCl_2$/ml at 0°, with stirring for a few minutes, yielded after centrifugation for 10 min at 30,000 *g* a clear supernatant ("nuclear" fraction).

Data on the activity of the supernatant and "nuclear" fractions from noninfected and infected protoplasts, under the conditions of the standard assay, are shown in Table 1. It may be seen that there was little or no activity in either fraction before infection with the RNA phage. After infection the supernatant and "nuclear" fractions had similar specific activity. However, the total amount of enzyme was larger in the former than in the latter for it contained 2–4 times more protein. It may also be seen from Table 1 (experiment 1) that, when the assay was conducted

TABLE 1

EFFECT OF RNA PHAGE INFECTION ON THE INCORPORATION OF NUCLEOTIDES INTO RNA IN LYSATES OF *E. coli* PROTOPLASTS*

Experiment no.	Fraction	Uninfected Protoplasts Assay		Infected Protoplasts Assay	
		Standard	No DNAase (DNA added)†	Standard	No DNAase (DNA added)†
1‡	Supernatant	0	80	107	168
	"Nuclear"	29**	470**	105**	740**
2§	Supernatant	0	...	173	...
	"Nuclear"	0	...	113	...
3‡	Supernatant	0	...	156	...
	"Nuclear"	16	...	158	...

* Values expressed as μμmoles of GMP-C^{14} incorporated/min/mg protein.
† 60 μg of calf thymus DNA.
‡ Protoplasts prepared in the presence of 0.005 *M* Mg^{++}.
§ Protoplasts prepared in the absence of Mg^{++}.
** Assays carried out on a suspension of the "nuclear" fraction not treated with DNAase.

in the absence of DNAase and in the presence of added DNA, both fractions exhibited higher GMP-C^{14} incorporation activity both before and after infection. The additional nucleotide incorporation under these conditions reflects the activity of the DNA-primed RNA polymerase. It is apparent that the "nuclear" fraction contained more RNA polymerase, and of much higher specific activity, than the supernatant.

Eighty per cent of the RNA synthetase activity of the 30,000 *g* supernatant from infected protoplasts could be sedimented by centrifugation for two hr at 105,000 *g*. This indicates that the activity was associated with large molecules or particles. The specific activity of the resuspended precipitate was only slightly lower than that of the original supernatant.

Purification: All operations were conducted at 0°–5°. Thirty liters of culture of infected protoplasts yielded 567 ml of 30,000 *g* supernatant with 2.07 gm of protein.

Step 1. Extraction: As described above.

Step 2. Protamine fractionation: To the supernatant were added 11 ml of *M* succinate buffer, pH 6.3 0.03 ml of a one per cent DNAase solution and, after 10 min, 103 ml of one percent protamine sulfate (0.5 mg of protamine sulfate/mg protein) in 0.02 *M* succinate buffer, the solution having been adjusted to pH 6.3. After stirring for 10 min, the precipitate was collected by centrifugation and the supernatant discarded. The precipitate was homogenized with 91 ml of five per cent ammonium sulfate in 0.02 *M* Tris HCl buffer (pH adjusted to 7.2) and the homogenate centrifuged for 30 min at 80,000 *g* in the No. 30 rotor of the Spinco preparative ultracentrifuge. The sediment was re-extracted twice with a total of 42 ml of ten per cent ammonium sulfate in 0.02 *M* Tris HCl buffer adjusted to pH 7.2, and the pooled extracts centrifuged for 10 min at 30,000 *g* to remove insoluble material. This yielded 43 ml of opalescent solution with 240 mg of protein.

Step 3. Ammonium sulfate fractionation: To the above supernatant was added with mechanical stirring 0.1 gm of solid, finely powdered ammonium sulfate/ml. After stirring for 20 min the mixture was centrifuged for 20 min at 30,000 *g* and the precipitate was discarded. A further 0.075 gm of ammonium sulfate/ml was added to the supernatant as above. The precipitate was collected by centrifugation and dissolved in 0.02 *M* Tris HCl buffer, pH 7.2, to give 24 ml of solution with 48 mg of protein.

Step 4. Removal of inactive proteins by adsorption on calcium phosphate gel: The opalescent solution from the above step was adjusted at 0° to pH 8.2 by addition of 0.28 ml of 1.0 *M* Tris. To this solution was added 7.8 ml of $Ca_3(PO_4)_2$ gel (32 mg $Ca_3(PO_4)_2$/ml) and, after mixing rapidly, the mixture was immediately centrifuged at 25,000 *g* for five minutes. The supernatant solution, 28 ml, contained 18 mg of protein. This solution was stored at $-12°$ for several days.

Steps 5 and 6. Deoxycholate treatment: To a 10 ml aliquot of the solution from the previous step (6.4 mg of protein) was added 1 ml of ten per cent sodium deoxycholate. After 30 min the solution was centrifuged at 26,000 *g* in the No. 40 rotor of the Spinco preparative ultracentrifuge. To the supernatant was added at once 0.4 ml/ml of 0.2 *M* $MgSO_4$; this addition was made dropwise with vigorous stirring. The copious precipitate was removed by centrifugation at 30,000 *g* for ten minutes and discarded. The supernatant, 15.0 ml, contained 0.7 mg of protein.

A summary of the purification procedure is given in Table 2.

TABLE 2

PURIFICATION OF RNA SYNTHETASE FROM LYSATES OF INFECTED *E. coli* PROTOPLASTS*

	Volume (ml)	Protein (mg)	Units†	Specific activity‡	Yield (%)
1. 30,000 *g* supernatant	567	2,070	99	0.048**	100
2. Protamine fractionation	43	240	58	0.24	58
3. $(NH_4)_2SO_4$ fractionation	24	48	41	0.85	41
4. $Ca_3(PO_4)_2$ gel supernatant	28	18	27	1.50	27
4. $Ca_3(PO_4)_2$ gel supernatant§	10	6.4	4.5	0.70	100
5. Deoxycholate supernatant	11	3.6	2.5	0.69	56
6. $MgSO_4$ supernatant	15.0	0.7	1.7	2.42	38

* From 30 liters of culture.
† mμmoles of GMP-C^{14} incorporated/min.
‡ Units/mg protein.
§ Part of step 4 fraction after storage at −12° for four days.
** The specific activity at this stage varies from preparation to preparation. It is generally higher (above 0.1) in lysates from one or two liters of culture.

Properties of Enzyme.—The enzyme was very unstable during the early purification steps and lost activity rapidly. As may be seen from Table 2, the enzyme was still unstable at step 4 of purification; it lost 50 per cent of its activity on storage for four days at −12°.

The enzyme remained particulate throughout the purification thus far achieved. At step 6 (Table 2), centrifugation of the $MgSO_4$ supernatant for two hours at 105,000 *g* sedimented 80–100 per cent of the activity. The solution of this precipitate had about the same specific activity as the $MgSO_4$ supernatant. Its light absorption ratio 280 mμ/260 mμ was below 0.6, indicating the presence of considerable amounts of nucleic acid.

Most of the RNA polymerase originally present in the 30,000 *g* supernatant was removed on purification of the synthetase. Thus, in one experiment the specific activity of the 30,000 *g* supernatant was 0.05 in the standard assay and 0.6 when assayed in the absence of DNAase and in the presence of added DNA, the difference representing the activity of the DNA-primed RNA polymerase. The corresponding step 6 fraction (prepared without using DNAase in step 2) that had been stored for several days had a specific activity of 1.6 in the former and 1.8 in the latter assay. On the other hand, polynucleotide phosphorylase—as assayed through the incorporation of AMP from C^{14}-labeled adenosine diphosphate—was purified to a greater extent than the synthetase. However, 0.2 *M* inorganic phosphate completely inhibited the phosphorylase activity of a step 4 preparation while the incorporation of GMP from GTP-C^{14}, in the standard synthetase assay, was inhibited only 17 per cent. The phage-induced RNA synthetase is therefore clearly distinct from RNA polymerase and polynucleotide phosphorylase.

Contrary to the DNA-primed RNA polymerase, which is strongly inhibited by actinomycin D,[9, 16] the RNA synthetase activity was not affected. In one experiment the specific activity at step 2 (Table 2) was 0.17 whether in the absence or presence of 24 μg of actinomycin D/ml.

Table 3 shows data on the incorporation of C^{14}-labeled nucleotides from each of the four ribonucleoside triphosphates and the effect of the presence of the other three nonlabeled triphosphates thereon. It may be seen that the presence of all four nucleoside triphosphates markedly increased the incorporation of each nucleotide.

Nature of Product.—Nucleic acid was isolated after incubation of a sample under

TABLE 3

INCORPORATION OF VARIOUS NUCLEOTIDES INTO RNA WITH RNA SYNTHETASE*

C^{14}-labeled ribonucleoside triphosphate added	Nonlabeled ribonucleoside triphosphates added	Specific activity of enzyme†
ATP	None	0.22
ATP	GTP, UTP, CTP	0.47
UTP	None	0.09
UTP	ATP, GTP, CTP	0.36
CTP	None	0.11
CTP	ATP, GTP, UTP	0.36
GTP	None	<0.05
GTP	ATP, UTP, CTP	0.56

* Step 4 (Table 2) enzyme. The fraction had been kept frozen for several days and had lost some activity. Conditions of standard assay.
† mμmoles of labeled nucleotide incorporated/min/mg protein.

the conditions of the standard assay, except that the scale was larger (step 6 enzyme, 240 μg of protein; final volume, 8 ml) and that the nucleoside triphosphate mixture consisted of ATP-C^{14} (4 μmole, 2.9×10^6 cpm/μmole) with nonlabeled GTP, UTP, and CTP. After precipitation and washing three times with 0.4 *N* perchloric acid, the precipitate was suspended in 1.0 ml of water, brought into solution by addition of dilute ammonium hydroxide, and the pH adjusted to 7.2 with 0.02 *M* Tris-HCl buffer of the same pH. The total radioactivity of the solution was 8,750 cpm. The nucleic acid was extracted with phenol,[17] the aqueous phase made 0.1 *M* with respect to potassium chloride, and the nucleic acid precipitated by addition of two volumes of ethanol. After standing for two hours at 0°, the precipitate was collected by centrifugation and dissolved in 0.02 *M* Tris HCl buffer, pH 8.8, to make 1.6 ml solution. The total absorbancy of the solution at wavelength 260 mμ was 70 and its radioactivity 7,080 cpm.

A 0.8 ml aliquot of the above solution (total absorbancy, 35; 3,540 cpm) was incubated at 37° with Tris HCl buffer, pH 8.8, 40 μmoles; $MgCl_2$, 40 μmoles; and snake venom phosphodiesterase, 200 μg, in a final volume of 1.6 ml. Samples (0.2 ml) were withdrawn at various time intervals, mixed with 1.8 ml of ice-cold 0.4 *N* perchloric acid and 0.01 ml of two per cent serum albumin, and centrifuged at 30,000 *g* for ten minutes. The absorbancy of the supernatant fluid at 260 mμ was determined as a measure of the release of acid-soluble nucleotides; the release of radioactivity was determined by difference between the total radioactivity of the sample and that remaining in the perchloric acid precipitate. For radioactivity determination the precipitate was dissolved in 1.0 *N* ammonium hydroxide. The results, given in Table 4, show that acid-soluble nucleotides and radioactivity, i.e., AMP-C^{14}, were released at about the same rate. This indicates that the AMP-C^{14} incorporated was uniformly distributed throughout the nucleic acid chains and suggests *de novo* synthesis rather than addition of AMP residues to the end of preformed RNA chains.

Another sample of AMP-C^{14} nucleic acid containing 40,000 cpm (prepared with step 3 enzyme) was dissolved in 1.0 ml of 0.4 *N* potassium hydroxide and incubated for 12 hr at 37°. Over 99 per cent of the radioactive material became acid-soluble under these conditions. The solution was neutralized with perchloric acid and the potassium perchlorate removed by centrifugation. An aliquot (19,500 cpm) of the neutralized solution, with added cold adenosine (total absorbancy, 500) as carrier, was chromatographed according to Cohn[18] on a Dowex-1-formate (two per cent

TABLE 4

DIGESTION OF PRODUCT OF ENZYMATIC REACTION WITH SNAKE VENOM PHOSPHODIESTERASE

Incubation time (min)	Absorbancy of acid-soluble nucleotides	Radioactivity of acid-soluble nucleotides (cpm)
5	0.2	0
10	0.3	0
20	0.7	32
50	1.2	83
128	2.5	136
480	4.6	325*

* All of the radioactive material was rendered acid-soluble at this time.

cross-linked) column. The recovery of radioactivity was 15,000 cpm of which 12,000 cpm were in the pooled AMP fractions and 3,000 cpm in the adenosine fraction. These results identify the AMP-C^{14}-containing product as RNA and show once more that the AMP was incorporated predominantly in nonterminal positions.

Discussion.—As already noted, the enzyme preparations contain considerable amounts of nucleic acid, presumably RNA. Attempts to remove this endogenous RNA without denaturing the enzyme have thus far been unsuccessful. Therefore, it has not been possible to demonstrate a dependence on added RNA for activity of the enzyme. Nevertheless, the results as a whole suggest that the enzyme may be concerned with the replication of phage RNA. While the RNA might be removed on further purification, the possibility that the enzyme might be a nucleoprotein with firmly bound primer RNA should be kept in mind. Since the viral RNA has a molecular weight of about 700,000[14] a nucleoprotein containing this RNA could have a sedimentation coefficient in the 30 *S* range.

Since the enzyme is particulate it is of some interest that Nathans *et al.*[19] observed the incorporation of histidine-C^{14} into particle-bound protein by a cell-free protein synthesizing system of *E. coli* with f_2 coliphage RNA as messenger. As the viral coat protein contains no histidine and was released into the supernatant during amino acid incorporation by this system, it may be that the particle-bound protein may in part consist of the virus-induced RNA synthetase described in this paper.

Summary. – Infection of *E. coli* with an RNA phage induces the formation of an enzyme catalyzing the incorporation of nucleotides from ribonucleoside 5′-triphosphates into RNA. This enzyme was purified about 50-fold and appears to be particulate in nature. Optimal activity requires the presence of all four ribonucleoside triphosphates, ATP, GTP, UTP, and CTP. The purified preparations are essentially free of DNA-dependent RNA polymerase but contain polynucleotide phosphorylase. They also contain large amounts of RNA and a dependence on added RNA for activity of the enzyme has not been shown. The enzyme, for which the name RNA synthetase is suggested, may be involved in the replication of viral RNA.

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[1] Abbreviations: RNA and DNA, ribonucleic and deoxyribonucleic acid; ATP, GTP, UTP, and CTP, the ribonucleoside 5′-triphosphates of adenosine, guanosine, uridine, and cytidine; GMP, guanosine monophosphate; AMP, adenosine monophosphate; Tris, tris(hydroxymethyl)-aminomethane; RNAase, pancreatic ribonuclease; DNAase, pancreatic deoxyribonuclease.

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*ON THE ROLE OF HISTONES IN REGULATING RIBONUCLEIC ACID SYNTHESIS IN THE CELL NUCLEUS**

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The experiments to be described in this paper are concerned with the role of histones in nuclear function and chromosome structure. They deal with RNA synthesis in isolated nuclei or in chromatin strands, with the effects of different histones on this and other biosynthetic processes, and with the enhancement of RNA synthesis in histone-depleted nuclei.

We have shown previously that the addition of histones to isolated nuclei, or to nuclear ribosomes, inhibits or represses a number of their biosynthetic activities.[1–9] For example, one can observe a decrease in the rate of incorporation of C^{14}-labeled amino acids into protein[1–5] or of C^{14}-adenosine into RNA[5, 7–9] when isolated thymus nuclei are given a supplement of basic proteins such as histones, protamines or polylysine. The inhibition observed is due in part to the fact that added histones can inhibit nuclear ATP synthesis[9] and in this way prevent the ATP-dependent "activation" of amino acids needed for protein synthesis[10, 11] and also diminish the kinase activities and ATP "pool" required for RNA synthesis.[12, 13, 6] Such indirect effects are not surprising, considering that histones are known to have a very broad spectrum of inhibitory actions due to their ability to form complexes with many

enzymes (e.g., cytochrome oxidase[14]) or with their substrates or necessary cofactors. Indeed, the toxicity of the histones has been demonstrated in intact, living cells.[15, 16]

Because of the easy combination between the positively-charged histones and negatively-charged polymers, DNA in particular, one would also expect the addition of histones to influence the enzymatic synthesis of polynucleotides by complexing with the necessary DNA "primers."[6, 13, 17, 18] In this connection, the inhibition of RNA synthesis in thymus nuclei by thymus histones has already been described,[5–7] and recent elegant experiments by Huang and Bonner have shown that DNA-histone complexes fail to act as "primers" for RNA synthesis in the presence of an RNA polymerase from pea seedling nuclei.[19]

Is the repression of RNA synthesis *in vitro* by added histones an indication of their true biological function, or is it simply another instance of their general capacity to combine with and inhibit complex enzyme systems?

The experiments to be described below deal with this question. They offer some new and direct indications that histones do play a role in the regulation of nuclear RNA synthesis and that the mechanism of this regulation involves more than a simple inhibition. Two types of evidence are presented; the first compares different histones for their effects on the synthesis of nuclear RNA, DNA, and protein. The second approach is based on the removal of histones from the nucleus and shows: (1) that histone-depleted nuclei have an increased rate of RNA synthesis, and (2) that the newly-formed RNA includes "messenger"-RNAs which differ in composition from the bulk of the RNA in the thymocyte nucleus. The implications of these findings for chromosome function in differentiated cells are discussed.

Materials and Methods.—Isolation of nuclei: Nuclei were isolated from fresh calf thymus tissue after breaking the cells in hypotonic sucrose solution (0.20 *M* sucrose–0.003 *M* $CaCl_2$). The nuclei were then separated in 0.25 *M* sucrose as described elsewhere.[20–22] Most preparations contain fewer than 5 cells per 100 nuclei as judged by electron microscopy.

Preparation of histones: The *total histones* of thymus nuclei were prepared by acid extraction of nuclei previously washed with 0.1 *M* "tris" buffer (pH 7.6) to remove other soluble proteins and nuclear ribosomes.[23] The 0.1 *N* HCl extract was dialyzed briefly against 0.01 *N* HCl, then against water. About 32% of the nuclear dry weight is extractable in this way.

The *lysine-rich histones* were prepared by the method of Johns and Butler,[24] using isolated nuclei rather than whole tissue. The lysine-rich Histone Fraction II was isolated by the method of Daly and Mirsky.[25]

The *arginine-rich histones* were also prepared by the method of Johns and Butler,[24] starting with the isolated nuclei. The arginine-rich Histone Fraction I was isolated by isoelectric precipitation at pH 10.6.[25] Some arginine-rich and lysine-rich histones were prepared by chromatography on carboxymethyl-cellulose.[26]

All the above fractions were tested for their effects on nuclear synthetic reactions.

Effects of Added Histones on Synthetic Processes in the Nucleus.—Histone inhibition of RNA synthesis: The synthesis of ribonucleic acid in isolated calf thymus nuclei has been followed using adenine-8-C^{14}, adenosine-8-C^{14}, guanosine-8-C^{14}, orotic acid-6-C^{14}, and P^{32}-orthophosphate. The incorporation of these labeled compounds into nuclear RNA was allowed to proceed for 30 or 60 mins at 37° in the incubation medium described previously.[12, 22] Over 90 per cent of the counts incorporated can be removed by ribonuclease digestion,[12] or isolated in the appropriate nucleotides after KOH digestion and chromatography.[27, 28]

When histones are added to the incubation medium the synthesis of RNA is inhibited, the degree of inhibition depending on the type of histone and its concen-

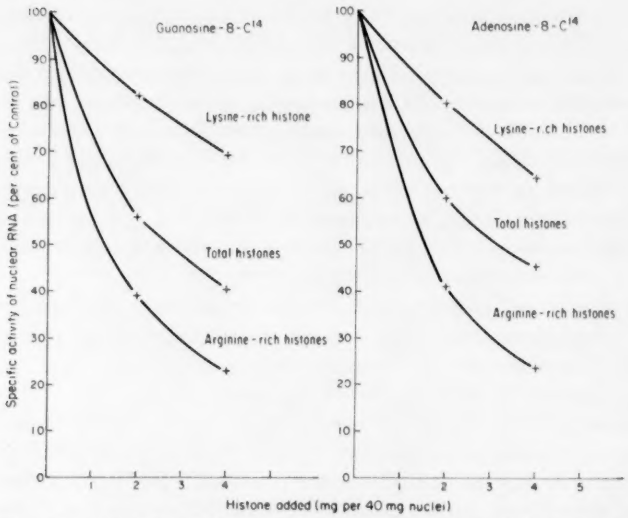

FIG. 1.—The effects of adding different histones on RNA synthesis in isolated thymus nuclei. The amount of histone added is plotted against the relative specific activity of the total nuclear RNA.

tration. The arginine-rich and lysine-rich fractions behave very differently in this regard. Figure 1 summarizes some results obtained using C^{14}-guanosine and C^{14}-adenosine as RNA precursors. It can be seen that the arginine-rich histones are strongly inhibitory while the lysine-rich fractions are comparatively ineffectual. The curve for inhibition by the total histone fraction, as expected, lies between these two extremes. Similar results are shown for orotic acid and adenine incorporation in Table 1. It is of interest that all preparations of lysine-rich histones tested were relatively weak inhibitors of nuclear RNA synthesis, although polylysine itself is a good inhibitor (Table 1).

Because histones also inhibit the "transport" of RNA precursors into nuclear "pools,"[8] other tests were carried out to show that the observed inhibition is not due to this effect, and it was found that the utilization of C^{14}-adenosine already in the nuclear "pool" was strongly inhibited by arginine-rich histones.

Histones also influence nuclear RNA synthesis by affecting nuclear energy metabolism. This aspect of histone action is discussed in detail elsewhere,[9, 20] but it should be noted here that the arginine-rich histones are more inhibitory than are the lysine-rich. The inhibition of nuclear ATP synthesis by added histones has many consequences, since it restricts the energy supply needed for the synthesis of RNA, DNA, and protein.

Effects of added histones on nuclear protein synthesis: It has been shown that histones inhibit amino acid incorporation by isolated nuclei[1, 3, 5] and by nuclear ribosomes.[4] A comparison of the different fractions again shows that the lysine-rich histones are comparatively ineffectual, while the arginine-rich histones are strong inhibitors of amino acid uptake.[1] The extent of inhibition is usually as great or greater than the corresponding repression of nuclear RNA synthesis.

Because alanine-1-C^{14} incorporation depends so largely on its "transport" into

TABLE 1

EFFECTS OF DIFFERENT HISTONES AND POLYLYSINE ON RNA SYNTHESIS IN THYMUS NUCLEI

Histone added	mg	Specific Activity of Nuclear RNA after Labeling with: Adenosine-8-C^{14} cpm/mg	% inhibition	Guanosine-8-C^{14} cpm/mg	% inhibition	Orotic acid-6-C^{14} cpm/mg	% inhibition	Adenine-8-C^{14} cpm/mg	% inhibition
Control—no histone	0	1295	...	2465	...	1900	...	967	...
Total histone	1	1049	19.0	1738	29.5	...	...	...	...
"	2	725	44.0	1380	44.0	...	...	...	...
"	4	531	59.0	998	59.5	...	...	...	...
Lysine-rich fraction*	1	1153	11.0	2206	10.5	...	...	916	5.3
"	2	1036	20.0	2021	18.0	1880	1.1	889	8.1
"	4	829	36.0	1700	31.0	1925	0	867	10.3
Lysine-rich fraction†	4	1210	6.6	...	...	...	...	...	...
Polylysine	4	715	44.8	...	...	...	...	...	...
Arginine-rich fraction*	1	803	38	...	...	...	...	...	...
"	2	505	61	...	...	1550	18.4	796	17.7
"	4	298	77	...	...	1390	26.8	542	44.0
Arginine-rich fraction†	4	540	58.3	567	77.0	...	...	...	...

* Prepared by the method of Johns and Butler.[24]
† Prepared by the method of Daly and Mirsky.[25]

nuclear "pools"[8] (a reaction inhibitable by histones[8]), some experiments were carried out in which the amino acid was admitted to the "pool" before adding the histone supplement: alanine incorporation into protein was still inhibited.

Effects of histones on thymidine incorporation into DNA: Isolated thymus nuclei incorporate C^{14}-thymidine into DNA.[31, 6] The addition of the arginine-rich Histone I Fraction (1 mg/ml) reduced uptake from 16 c.p.m. per mg DNA in controls to 5.7 c.p.m. per mg DNA in histone-treated nuclei.

Some effects of removing histones from isolated nuclei—Method: Because the addition of histone supplements to nuclei produces such a wide range of inhibitions, it was decided to approach the problem in a new way—by removing the histones selectively and testing for changes in function or activity of the histone-depleted nuclei. One procedure we have used makes use of trypsin because of its known preferential hydrolysis of peptide bonds involving arginine and lysine. When trypsin is added to nuclear suspensions (at levels of 0.5 mg per ml; 1 mg enzyme per 40 mg nuclei), much of the histone is hydrolyzed and released. The extent of the hydrolysis can be followed roughly by Millon analyses[32] of the acid-soluble proteins which remain in the trypsin-treated nuclei. About 70 per cent of the total histone is removed in 30 min at 37°, while the losses of nonhistone protein are comparatively small.

Enhancement of RNA synthesis: This treatment results in a marked stimulation of nuclear RNA synthesis. Some results are summarized in Table 2, which shows that the uptake of orotic acid, adenine, guanosine, and P^{32}-orthophosphate are all much higher in histone-depleted nuclei than in the corresponding "controls." Stimulations ranging from 200–400 per cent are the rule. The increased radioactivity has been definitely localized in the nuclear RNA, which was extracted in hot 10 per cent NaCl, precipitated with ethanol, hydrolyzed in KOH and chromatographed on Dowex-1 to separate the component nucleotides (Table 2).

TABLE 2

STIMULATION OF RNA SYNTHESIS IN ISOLATED THYMUS NUCLEI FOLLOWING HISTONE REMOVAL BY TRYPSIN

Conditions of experiment	Precursor added	Specific Activity of RNA Nucleotides: Total nucleotides cpm/mg	Adenylic acid cpm/μmole	Cytidylic acid cpm/μmole
Nuclei—alone	Orotic-6-C^{14} acid	74.1	...	...
" + 0.5 mg trypsin	" "	157.	...	...
" + 1.0 mg trypsin	" "	330.	...	...
Nuclei—alone	Adenine-8-C^{14}	330.	97.	...
" + 1.0 mg trypsin	" "	887.	256.	...
Nuclei—alone	Guanosine-8-C^{14}	663.	...	...
" + 1.0 mg trypsin	" "	1390.	...	...
Nuclei—alone	P^{32}-orthophosphate	1388.	9,380.	6,400.
" + 1.0 mg trypsin	" "	3330.	26,070.	18,860.

Because trypsin digestion not only releases histones but also hydrolyzes some other proteins of the nucleus, inhibitory effects are seen in other synthetic systems. Amino acid incorporation usually drops by about 60 per cent.

Trypsin digestion disrupts nuclear structure (see below). To avoid this structural complication, the enzyme action was moderated by the addition of the crystalline trypsin inhibitor from soy bean.[33] When 0.8 mg of inhibitor was added per mg of enzyme, the nuclei remained intact (Fig. 3, *B*) while histone digestion proceeded due to the trypsin excess. Nuclear RNA synthesis was greatly enhanced, as shown in Figure 2, which plots the specific activity of nuclear RNA against the time of incubation. It can be seen that trypsin-treated nuclei (curve 3) are far more active in incorporating C^{14}-adenine and guanosine than the corresponding

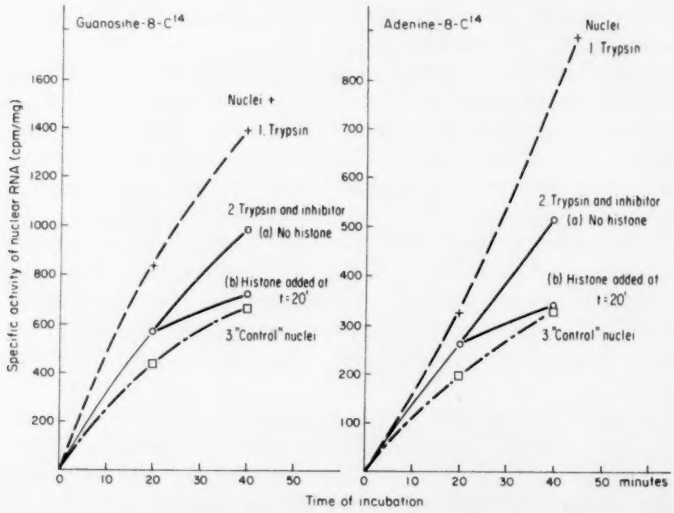

FIG. 2.—The effect of removing histones on nuclear RNA synthesis. The incorporation of guanosine-8-C^{14} and adenine-8-C^{14} is plotted as a function of the time of incubation at 37°. The lower curve (curve 3) shows the time course of C^{14} uptake in control nuclei. The upper curve (curve 1) shows the uptake in nuclei which were incubated with trypsin to remove 70% of the total histones. Curve 2 (a) is for nuclei treated with trypsin + soy inhibitor to moderate the enzyme action. Curve 2 (b) shows that the addition of 4 mg of histone at t = 20′ again suppresses nuclear RNA synthesis.

"controls" (curve 1). Nuclei treated with trypsin plus the soy inhibitor show about a 70 per cent increase in their uptake of these RNA precursors (curve 2*a*). The figure also shows that when histone is added back to the histone-depleted nuclei at 20 min, the synthesis of RNA is again inhibited (curve 2*b*).

Increased synthesis of "messenger"-RNAs in histone-depleted nuclei: We have shown previously that thymus nuclei contain[34] and actively synthesize[6] an RNA fraction of base composition similar to that of thymus DNA. The isolation of this "messenger"-RNA fraction has been carried out following the procedure of Sibatani *et al.*[34] after a 30 min incubation in the presence of P^{32}-labeled orthophosphate. In "control" nuclei the specific activity of the "messenger"-RNA fraction was 29,960 cpm per mg RNA-P; in trypsin-treated nuclei the corresponding figure was 90,150 cpm per mg RNA-P, an increase of over 300 per cent.

Some preliminary data on the distribution of P^{32} in the RNA nucleotides suggests that the composition of the newly synthesized RNA differs from that normally being made in the thymus nucleus. Increased P^{32} uptakes into guanylic and uridylic acids have been observed, but it remains to be proved that the removal of histones has activated previously "repressed" DNA primers of different nucleotide composition.

Structural effects of trypsin treatment: When nuclei are incubated in the presence of trypsin alone, as in the above experiments, there are profound changes in structure. Macroscopically, the suspension forms a thick gel. Electron microscopy of the gel shows that it consists of thin strands of chromatin, of varying diameters and degrees of aggregation. The smallest strands are about 50 Å in diameter (Fig. 3, *C* and *D*). If the incubation is carried out using trypsin plus trypsin inhibitor, gel formation does not occur, though much of the histone is digested. The nuclei after digestion appear normal, as judged by light and electron microscopy. (Compare Fig. 3, *B* with the untreated nuclei shown in Fig. 3, *A*.) It is a remarkable fact that nuclear structure can be disrupted by trypsin and still permit RNA synthesis to continue, and at a higher rate.

Discussion.—The evidence presented above indicates that histones can inhibit or repress a large number of nuclear biosynthetic activities, including RNA synthesis. The mechanism of the inhibition is complex and may involve several steps in the utilization of a precursor, beginning with transport and including ATP-dependent kinase reactions as well as the final polymerase reaction. The arginine-rich histones are strong inhibitors while the lysine-rich fractions are weakly inhibitory. These findings may be tentatively combined with earlier observations that most of the DNA in the thymus nucleus, or in other highly differentiated cell types, is "inactive" or "repressed" and can be removed, while the remaining DNA governs RNA synthesis.[6] One could suggest a model of chromosome structure in which most of the DNA was bound to histone fractions, such as the arginine-rich histones, which inhibit the synthesis of "messenger"-RNAs. Active genes or "priming" sites would contain the lysine-rich histone fractions or a histone complex which favors DNA utilization as a primer for RNA synthesis. Release of the arginine-rich histones would be expected to result in increased "messenger"-RNA synthesis and lead to the production of previously repressed "messages." The experimental data presented in this paper are in accord with such a model.

Summary.—The addition of thymus histones to isolated thymus nuclei inhibits

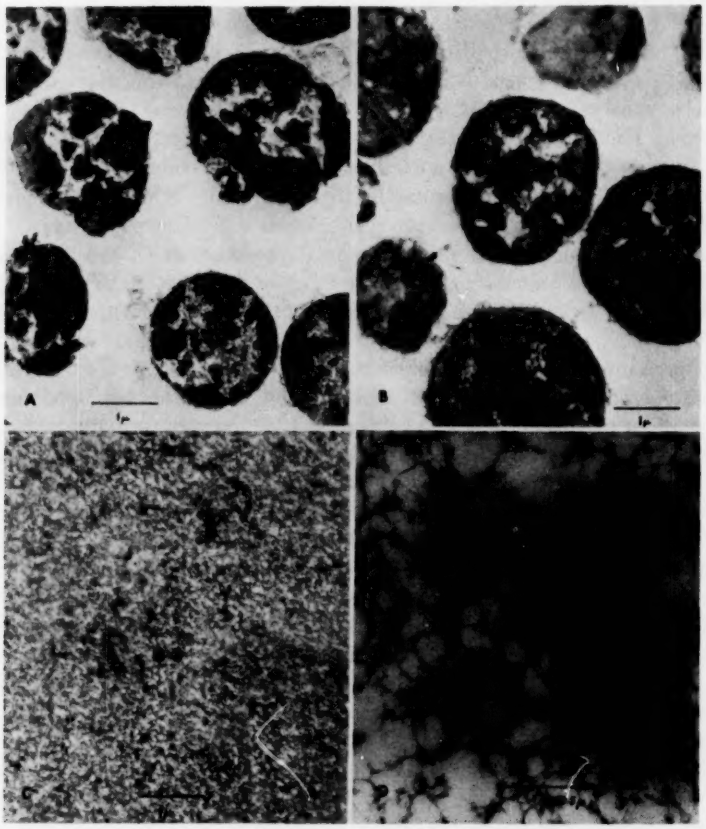

FIG. 3.—Electron micrographs of calf thymus nuclei before and after trypsin digestion.
A: Control nuclei, incubated for 20 min in the absence of trypsin.
B: Nuclei incubated with 1 mg trypsin plus 0.8 mg trypsin inhibitor for 20 minutes at 37°.
C: Gel remaining after trypsin treatment of nuclei.
D: Same as C, at higher magnification.

many biosynthetic reactions, including RNA synthesis. The arginine-rich histones are strong inhibitors; the lysine rich histones are relatively inert. Selective removal of the histones from the nucleus results in increased rates of RNA synthesis. The newly synthesized RNA occurs in the "messenger"-RNA fraction and may differ in its base composition from the usual "messenger"-RNA complement of the thymus. Some implications for chromosome structure and function are discussed.

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GENETICS: A CORRECTION

The classification *Biochemistry* for the article entitled "Interaction of Genotypes Determining Viability in *Drosophila busckii*," by R. C. Lewontin and Yoshiro Matsuo, which was published in the February issue of these PROCEEDINGS (vol. 49, no. 2, pp. 270–278), should have been *Genetics*.

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